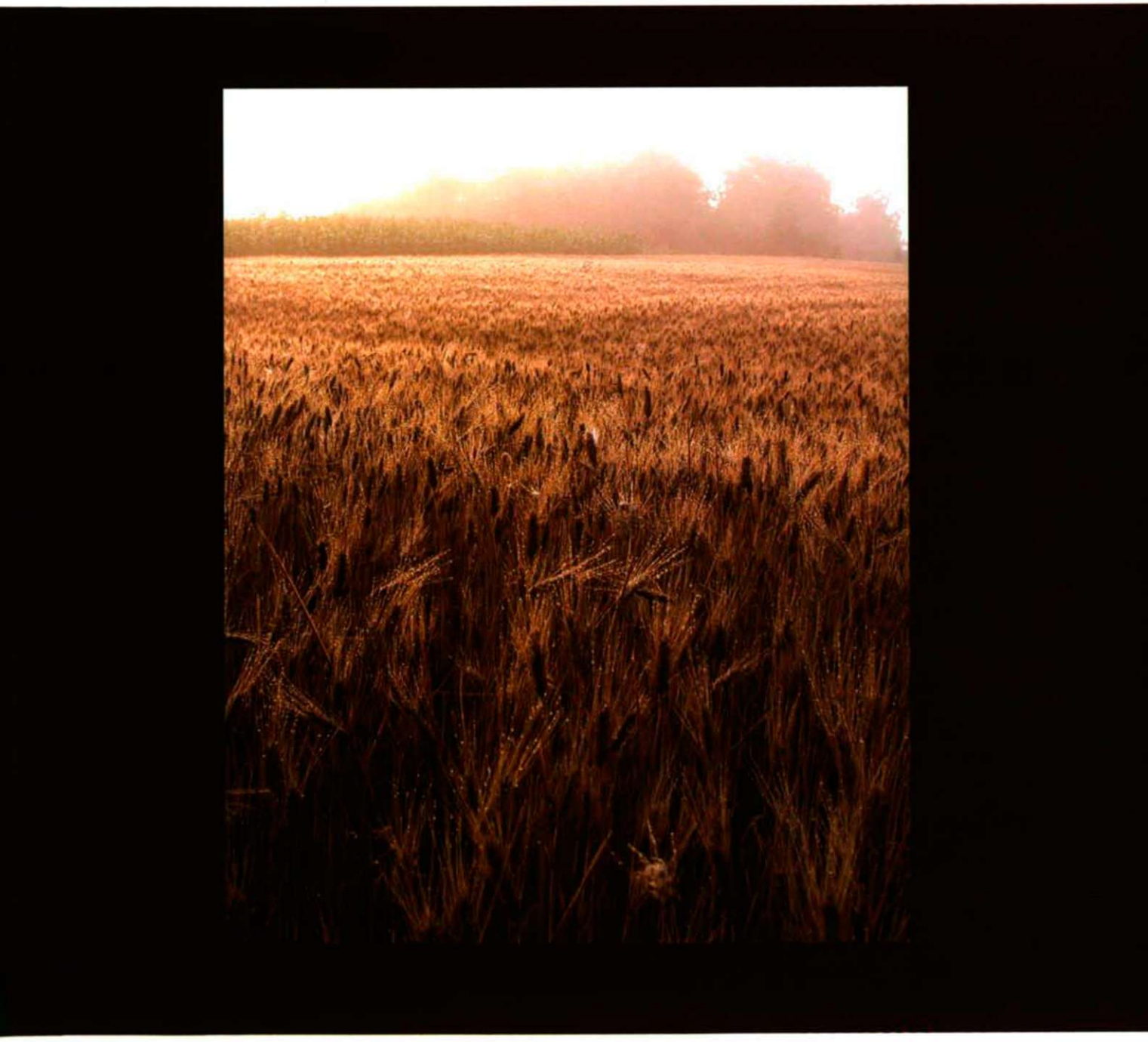
Acta Universitatis Szegediensis

Acta Biologica Szegediensis

Volume 53, Number 1, 2009



University of Szeged, Szeged, Hungary

Acta Biologica Szegediensis

Acta Biologica Szegediensis (ISSN 1588-385X print form; ISSN 1588-4082 online form), a member of the Acta Universitatis Szegediensis family of scientific journals (ISSN 0563-0592), is published yearly by the University of Szeged. Acta Biologica Szegediensis covers the growth areas of modern biology and publishes original research articles and reviews, involving, but not restricted to, the fields of anatomy, embryology and histology, anthropology, biochemistry, biophysics, biotechnology, botany and plant physiology, all areas of clinical sciences, conservation biology, ecology, genetics, microbiology, molecular biology, neurosciences, paleontology, pharmacology, physiology and pathophysiology, and zoology. Occasionally, Acta Biologica Szegediensis will publish symposium materials. Acta Biologica Szegediensis particularly encourages young investigators and clinicians to submit novel results of interest.

Submission of manuscripts

Manuscripts should be prepared in accordance with the Instructions to Authors published in each issue, also available at http://www.sci.u-szeged.hu/ABS, and submitted to:

Károly Gulya
Acta Biologica Szegediensis, Editorial Office
Department of Cell Biology and Molecular Medicine
University of Szeged
4 Somogyi u., H-6720 Szeged, Hungary
Phone: 36 (62) 544-570, fax: 36 (62) 544-569
E-mail: gulyak@bio.u-szeged.hu

Correspondence relating to the status of the manuscripts, proofs, publication, reprints and advertising should be sent to:

Tamás Mikola
Acta Biologica Szegediensis, Editorial Office
Department of Cell Biology and Molecular Medicine
University of Szeged
4 Somogyi u., H-6720 Szeged, Hungary
Phone: 36 (62) 544-569, fax: 36 (62) 544-569
E-mail: zool@bio.u-szeged.hu

Subscriptions

Acta Biologica Szegediensis is published yearly in two issues per volume. All subscriptions relate to the calendar year and must be pre-paid. The annual subscription rate is currently 100 USD and includes air mail delivery and handling.

Acta Biologica Szegediensis is indexed in BIOSIS Database, EMBASE, Excerpta Medica, Elsevier BIOBASE (Current Awareness in Biological Sciences) and Zoological Record.

The Table of Contents for the current issue and those for previous issues can be found at http://www.sci.u-szeged.hu/ABS.

ACTA BIOLOGICA SZEGEDIENSIS

VOLUME 53(1), 2009

Table of Contents

Articles

On the cover: Wheat field at dawn. See in this issue Guóth et al. (2009) Acta Biol Szeged 53(1):1-7. Photo: Dr. László Erdei

Volume 53(1):1-7, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Chlorophyll *a* fluorescence induction parameters of flag leaves characterize genotypes and not the drought tolerance of wheat during grain filling under water deficit

Adrienn Guóth[1]*, Irma Tari[1], Ágnes Gallé[1], Jolán Csiszár[1], Ferenc Horváth[1], Attila Pécsváradi[1], László Cseuz[2] and László Erdei[1]

[1]Department of Plant Biology, University of Szeged, Szeged, Hungary, [2]Cereal Research Non-Profit Company, Szeged, Hungary

ABSTRACT Chlorophyll *a* fluorescence induction parameters of two drought tolerant (MV Emese and Plainsman V) and two sensitive (GK Élet and Cappelle Desprez) wheat cultivars were compared on the day 16 post anthesis (DPA) under control and water stressed conditions. It was found that under drought stress the chlorophyll *a* (Chl) content declined earlier in the sensitive genotypes, but the rate of Chl loss was much higher in the tolerant cultivars and also in the controls of cv. Plainsman after 12 DPA. The *trans*-zeatin content, a transport form of cytokinins also significantly declined to 16 DPA in the sensitive GK Élet. Because cytokinins prevent Chl loss, it may be a cause of the decreased Chl level in this cv. under drought. Neither net CO_2 assimilation rate (P_N) nor other Chl *a* fluorescence induction parameters such as Fv/Fm or Φ_{PSII}, qP and NPQ as a function of increasing photon flux densities (PFD) exhibited a special change which could characterize only tolerant or sensitive cultivars. Instead, these changes characterized the genotypes. In cvs MV Emese and Plainsman the flag leaf senescence could result in faster and better remobilization of the pre-stored carbon from vegetative tissues, and ultimately better yield than in GK Élet and Cappelle Desprez under drought. **Acta Biol Szeged 53(1): 1-7 (2009)**

KEY WORDS

Chlorophyll *a* fluorescence
drought tolerance
grain filling
wheat genotypes

Under the temperate zone early-summer droughts are increasingly frequent. This may severely limit grain yield since the water shortage coincides with the grain filling period of most cereals, including wheat, one of the main crops consumed by humans.

In grain filling period water deficit induces fast senescence in wheat, especially in older leaves. The primary expression of leaf senescence is the breakdown of chlorophyll and the decline of photosynthetic activity. It is generally accepted, that the genotypes which are able to sustain photosynthesis in flag leaf for longer time, tend to yield more (Richards 2000).

Since photosynthesis is one of the main metabolic processes determining crop production, chlorophyll fluorescence was used as an effective tool for monitoring the function of the photosynthetic apparatus in response to water stress (Flexas et al. 2002; Fracheboud and Leipner 2003; Zlatev and Yordanov 2004). The effect of drought on photosynthesis has long been controversial and it is still not clear whether chlorophyll *a* (Chl-*a*) fluorescence parameters are good indicators for drought sensitivity. Photosystem II (PSII) is sensitive to environmental limiting factors, but drought stress, as indicated by a number of studies with different plant species resulted in conflicting results.

Accepted June 10, 2009
*Corresponding author. E-mail: guotha@bio.u-szeged.hu

Water-limiting conditions probably have no significant effect on the primary photochemistry of PSII in wheat or durum wheat (Subrahmanyam et al. 2006; Hura et al. 2007) because the maximal quantum yield (variable/maximal fluorescence, Fv/Fm), the rapid fluorescence induction kinetics and polyphasic fluorescence transients were not significantly affected in dark-adapted leaves of six-week-old plants (Lu and Zhang, 1999). In contrast, the actual quantum yield of PSII electron transport (Φ_{PSII}) decreased, non photochemical quenching (NPQ) increased, but photochemical quenching coefficient (qP) did not change under water deficit in light adapted leaves in wheat (Lu and Zhang 1999; Hassan 2006; Subrahmanyam et al. 2006). It was found in other experiments that Fv/Fm decreased under drought stress conditions in young leaves (Hassan 2006) or was also reduced in flag leaves of wheat plants (Paknejad et al. 2007). Increase in the ground state fluorescence (Fo) or decrease in Fv/Fo and Fv/Fm can be considered to be reliable indicators for screening drought tolerance of barley genotypes (Li et al. 2006). Paknejad et al. (2007) also found that Fv, Fv/Fm and Fo had high correlation coefficients with drought sensitivity and with grain yield.

Yield is the most important economic trait, and grain production is the main selection criteria for drought resistance of wheat. Many environmental factors, such as high air or soil

temperature (Ugarte et al. 2007), time and application of fertilizers (Erdei et al. 1986), water or ozone stress (Fangmeier et al. 1994) may influence the productivity, and ultimately the grain yield.

It was found that yield components were infuenced by hormonal status of wheat plants under drought. Bernier (1988) suggested that the stress hormone, abscisic acid (ABA), which accumulates during water deficit, inhibited flowering, and ABA has also been shown to reduce leaf growth (Dodd et al. 1996). Another stimulus from the root system that may hasten flowering, may increase the number of kernels per ear and the cell number in the endosperm during early phase of the grain filling and ultimately may increase the grain yield, is cytokinin, which is transported from the root system to the shoot in the xylem. However, the synthesis in the root tip and transport to the shoot of cytokinin-like compounds has been shown to decrease due to drought (Davies et al. 1986). Furthermore, cytokinins may also delay the senescence of flag leaves.

Our aim was to compare the chlorophyll-*a* fluorescence induction parameters in drought tolerant and sensitive cultivars in well-watered and drought-stressed conditions during early phase of grain filling before the visible sign of leaf senescence and we were also interested whether these water stress-induced changes characterize the sensitive or tolerant cultivars or they characterize genotypes themselves.

Materials and Methods

Plant material and water stress treatment

Two Hungarian wheat cultivars, *Triticum aestivum* L. cv. MV Emese, a drought tolerant and cv. GK Élet, a drought sensitive genotype and two well-known standards, the drought tolerant Plainsman and the drought sensitive Cappelle Desprez have been investigated under well watered and drought stress conditions.

The experiments were carried out in the grain filling period. The plants were grown in plastic plots (3 plants per pot) containing a mixture of soil (type Terra, Hungary) and sand (1:1, v/v) at 300 µmol m^{-2} s^{-1}light intensity, 12/12 hour day/night illumination, at 26/20°C day/night temperature and 55-60% air humidity (Tari et al. 2008). From 4 days before the booting stage two watering schedules were applied. The first group of the plants, the control plants, received sufficient irrigation, while the other group was subjected to water stress (WS) treatment by withholding irrigation. Irrigation occurred every 2nd day to reach 60% of the total soil water capacity for control plants, and 25% for stressed plants. Samples were prepared and *in vivo* measurements were made on the 16th days post anthesis (DPA). At maturity, ears were harvested from five pots per variety and per treatment to determine the grain mass per ear, the number of grains per ear and the thousand grain mass. The experiments were repeated three times.

Pigment analyses

For pigment analysis a two step extraction was applied. The fully expanded flag leaves were homogenized in ice-cold 100% (v/v %) acetone (1,5 ml for 250 mg sample), and extracted for 24 hours. Samples were centrifuged at 5000 g for 15 minutes at 4°C. The pellet was extracted again with 80% (v/v %) acetone (1,5 ml for 250 mg sample) for 24 hours. After centrifuging (5000 g, 15 minutes, 4°C), the supernatants were collected. The pigment composition was measured by spectrophotometer according to Lichtenthaler and Wellburn (1983).

Measurement of chlorophyll fluorescence parameters

Chlorophyll-*a* (Chl *a*) fluorescence was measured on flag leaves with a portable photosynthesis system (LI-6400, LI-COR, Inc.; Lincoln, NE). After 20 minutes of dark adaptation the ground state fluorescence level (F_0) with all PSII reaction centres open was determined by a modulated measuring light, which was sufficiently low not to induce any significant variable fluorescence. The maximal fluorescence level (F_m) with all PSII centres closed was measured after 20 min of dark adaptation by 0,2 s saturating pulse at 8000 µmol m^{-2} s^{-1}. Then the leaf was continuously illuminated with white actinic light at an intensity of 500 µmol m^{-2} s^{-1}. After 20 minutes the steady-state value of fluorescence (Fs) was recorded and a second saturating pulse (8000 µmol m^{-2} s^{-1}) was imposed to determine the maximum fluorescence level (Fm') in light-adapted state. The actinic light was removed and the minimum fluorescence level in the light-adapted state (F_0') was determined by illuminating the leaf with 3 s far-red light (5 µmol m^{-2} s^{-1}). Light response curves were generated by varying light intensities (50, 150, 300, 400, 500, 700, 1200, 1500 µmol/m^2s) after 10 minutes of dark adaptation. The light curves measured on the 16th days after anthesis.

By using fluorescence parameters determined in both light- and dark-adapted leaves, the following calculation were made (1) the maximal quantum yield of PSII photochemistry, Fv/Fm, (2) the photochemical quenching coefficient, qP = (Fm'-Fs)/(Fm'-Fo') (Bilger and Schreiber 1986), (3) non-photochemical quenching NPQ=(Fm-Fm')/Fm' (Bilger and Björkman, 1990), (4) the actual quantum yield of PSII electron transport in the light adapted-state, Φ_{PSII} = (Fm'-Fs)/Fm' (Genty et al. 1989). The net photosynthetic rate (P_N) of the flag leaves as a function of photon flux density (PFD) was also determined.

Determination of trans-zeatine content of flag leaves

Samples of 1000 mg leaf tissues were ground and extracted for 4 hours at 0°C in 2,5 ml of 80% (v/v) methanol containing 1 mg butylated hydroxytoluene as an antioxidant. After

Table 1. The effects of soil drought on the chlorophyll-*a* and *b* contents in μg DW^{-1} $(g)^{-1}$ in flag leaves of wheat plants during grain filling period. (DPA: days post anthesis.)

Triticum aestivum L. cultivar	Treatment	Number of grains per ear	Mass of grains per ear (g)	1000-grain dry mass (g)
cv MV Emese	Control	27.05 ± 7,4	1.32 ± 0.54	52.57 ± 4.8
	Drought	19.63 ± 4.4** (-27.40%)	0.80 ± 0.1*** (-39.53%)	41.74 ± 12.2** (-21.22%)
cv. GK Élet	Control	31.6 ± 4.9	1.59 ± 0.3	50.32 ± 6.9
	Drought	17.76 ± 6.5*** (-43.78%)	0.38 ± 0.2 *** (-75.71%)	18.76 ± 1.9** (-62.71%)
cv. Plainsman	Control	17.72 ± 8.1	0.64 ± 0.4	40.83 ± 3.0
	Drought	15.80 ± 4.2 (-10.77%)	0.58 ± 0.1 (-9.41%)	39.58 ± 1.6 (-4.01%)
cv. Cappelle D.	Control	27.71 ± 8.2	1.80 ± 0.7	65.73 ± 16.7
	Drought	15.85 ± 4.5*** (-42.78%)	0.63 ± 0.4** (-64.57%)	34.83 ± 13.5*** (-47.00%)

centrifugation at 3000 rpm for 10 min, the pellet was extracted further in 7.5 ml of the same medium for 12 hours. After a second centrifugation, the supernatants were combined and the samples were purified by Discovery C18 Cartridge (Supelco, USA), pre-washed with 10 ml of 100% and 5 ml of 80% methanol. The hormone fractions were then eluted from the columns with 7.5 ml of 100% methanol and 7.5 ml of diethyl ether. The leaf extracts were evaporated to dryness and before quantification they were dissolved in Tris-buffered saline (TBS) (800 ml of dH2O, 3.03 g Tris, 5.84 g NaCl, 0,2 g $MgCl_2$ hexahydrate and 0.2 g Na azide, (pH 7.5; 2500 μl/1000 mg sample). *Trans*-zeatin riboside (ZR) was analysed by ELISA employing Phytodetek Assay Kit (Agdia) supplied by Sigma Ltd. Colour absorbancy following reaction with the substrate was read at 405 nm using a plate auto reader (Dynatech MR 4000). Percentage binding was calculated using established procedures (Weiler et al. 1981). The recovery was determined as described by Yang et al. (2003).

Statistical analysis

Data presented in the figures are means of three to five replications ± SD. Data from each sampling date were analyzed separately by Student's t-test using a SigmaStat 3.1 software. Means denoted by *, ** or *** were significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels, respectively.

Results

Plant water status

Witholding irrigation resulted in reduction of leaf water potential both in sensitive and tolerant genotypes and the reduction was more pronounced in GK Élet and Cappelle Desprez, the more sensitive cultivars. Under drought stress water potential (ψ_w) values were lower between 4 and 12 DPA in GK Élet and Cappelle Desprez than in MV Emese. In the sensitive cultivars ψ_w decreased and remained near -1,5, while in the more tolerant MV Emese ψ_w reached -1,3 just at 12 DPA. In Plainsman there was a slight decrease in ψ_w at 12 DPA under water deficit, but ψ_w of stressed and control plants also decreased dramatically until 24 DPA (Guóth et al. 2009).

Water stress (WS) reduced also the relative water content (RWC) in the sensitive genotypes and in MV Emese. In GK Élet and in Cappelle Desprez, the differences were significant between the well-irrigated and stressed plants from the booting stage to the end of the experiment, while in MV Emese no differences were observed until the 4 DPA. The RWC values in the stressed plants were much higher in MV Emese compared to GK Élet and Cappelle Desprez. In Plainsman, RWC values under WS decreased in parallel with the control (Gallé et al. 2009).

Chlorophyll and trans-zeatin riboside contents

Chlorophyll *a* and *b* (Chl-*a* and *b*) contents did not decrease markedly in response to water stress until 12 DPA and no significant changes were found between the controls and water stressed plants in Chl-*a* contents in case of tolerant cultivars cvs Emese and Plainsman. Chl-a contents decreased significantly in flag leaves exposed to WS in sensitive cultivars Élet and Cappelle and in Chl-*b* contents in case of Cappelle Desprez at 12 DPA (Table 1). In cv. Plainsman the senescence process of flag leaves began also earlier in control plants than in MV Emese and GK Élet. Between 12 and 24 DPA, however, the rate of Chl loss was much higher in the tolerant cultivars cvs Emese and Plainsman.

Trans-zeatin riboside, a transport form of citokinins, also accumulated in flag leaves. ZR concentration was 40-43% greater than that of zeatin and its concentration changed parallel with that of zeatin in wheat flag leaves during grain filling (Yang et al. 2003). In our experiments there were no significant differences in ZR content of flag leaves in control and droughted plants with the exception of cv. GK Élet, a

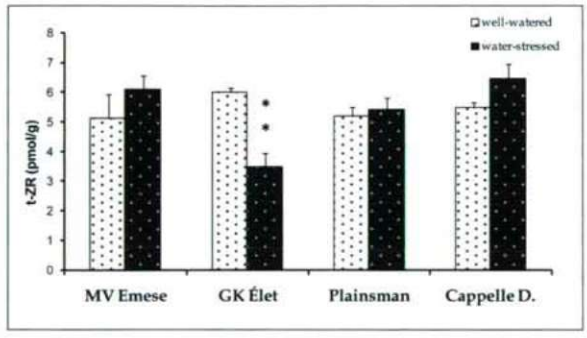


Figure 1. *Trans*-zeatin riboside contents in well-watered (o) and water-stressed (●) flag leaves at 16 DPA during grain filling in MV Emese, GK Élet, Plainsman and Cappelle Desprez wheat cultivars. Data are means ± SD of 3-5 independent samples. *, **, *** are for significant differences comparing to the control at 0.05, 0.01, 0.001 levels, respectively.

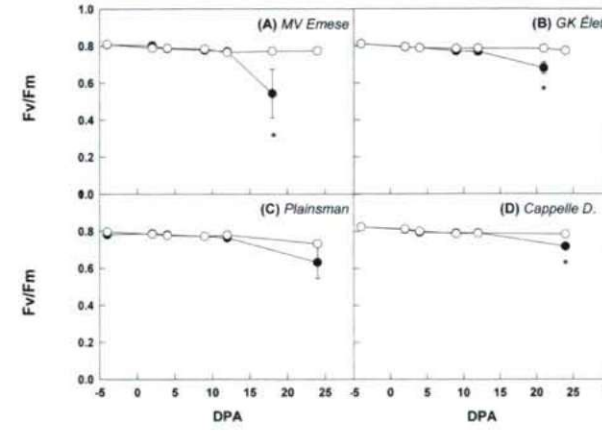


Figure 2. Changes of the maximal quantum yield of PSII photochemistry (Fv/Fm) in well-watered (o) and water-stressed (●) flag leaves during grain filling in MV Emese (A), GK Élet (B), Plainsman (C) and Cappelle Desprez (D) wheat cultivars. Data are means ± SD of 3-5 independent samples. SD bars are not shown where smaller than symbols. *, **, *** are for significant differences comparing to the control at 0.05, 0.01, 0.001 levels, respectively.

sensitive cultivar (Fig. 1). In this genotype, a significant reduction in ZR content occurred in parallel with a significant decrease of Chl-*a* contents (Table 1).

Chlorophyll *a* fluorescence and photosynthetic rate

Fv/Fm was measured on the flag leaves during whole grain filling period. The changes of Φ_{PSII}, qP, NPQ and P_N were determined on 16 DPA as a function of increasing PFD.

The values of maximal efficiency of PS II photochemistry (F_v/F_m) remained around 0.77-0.8 until the 12th day after anthesis in all varieties. Values decreased significantly under water deficit faster in the tolerant cv. MV Emese and in the sensitive GK Élet. In the two other cultivars Fv/Fm values decreased only on the last measuring days. In Plainsman, on the 24 DPA, Fv/Fm values decreased also in control plants (Fig. 2).

The Φ_{PSII}, qP, NPQ and P_N did not change considerably until 12 DPA under drought stress compared to the controls at 500 µmol m^{-2} s^{-1} light intensity in the investigated cultivars, and with the exception of Plainsman, the decreases in Φ_{PSII}, qP and P_N became significant on the last measuring days in the stressed plants. (Guóth et al. 2009). In case of cv Plainsman, these parameters changed parallel in control and water-stressed plants.

The responses of the actual P_N, Φ_{PSII}, qP and NPQ to different photon flux densities (PFDs) at 16 DPA are summarized in Figure 3.

In control plants the photosynthetic rate at light saturation (A_{max}) was highest in case of cv Élet, the other cultivars exhibited lower saturation values and severe photoinhibition at 1500 µmol m^{-2} s^{-1}. The net CO_2 fixation rate was limited under drought stress in three cultivars, among them cv. MV Emese was seriously affected, and interestingly, there were no significant changes between the P_N/PFD curves of control and water-stressed plants in case of the sensitive genotype, Cappelle Desprez, at 16 DPA.

In MV Emese the Φ_{PSII}, qP and at higher light intensities NPQ values differed significantly between control and stressed plants at all PFDs, this contrasts with the data found in cv. Cappelle Desprez where no differences were found at any PFDs. In cv. MV Emese Φ_{PSII} and qP markedly decreased under WS, and NPQ stopped increasing in the stressed plants above 700 µmol m^{-2} s^{-1} PFD. In GK Élet only slight differences can be observed in Φ_{PSII}, qP and NPQ from 700 µmol m^{-2} s^{-1}. In Plainsman qP decreased and NPQ increased significantly only at 1200 and 1500 µmol m^{-2} s^{-1} compared to the control (Figure 3).

Yield parameters

The number of grains per ear, the mass of grains per ear and the grain weight decreased during water deficit in MV Emese, GK Élet and Cappelle Desprez. The difference between the tolerant MV Emese and the sensitive varieties was significant, and the yield parameters of GK Élet and Cappelle-Desprez declined at higher rate than in MV Emese. In cv. Plainsman the crop yield did not decrease significantly under drought stress (Table 2).

Discussion

There are several physiological traits related to water stress, and scientists make considerable effort to find direct correlations between these parameters and grain yield to facilitate the screening and selection of cultivars for drought tolerance. A strong relationship between biomass at anthesis and yield has been demonstrated in bread wheat (van den Boogard et

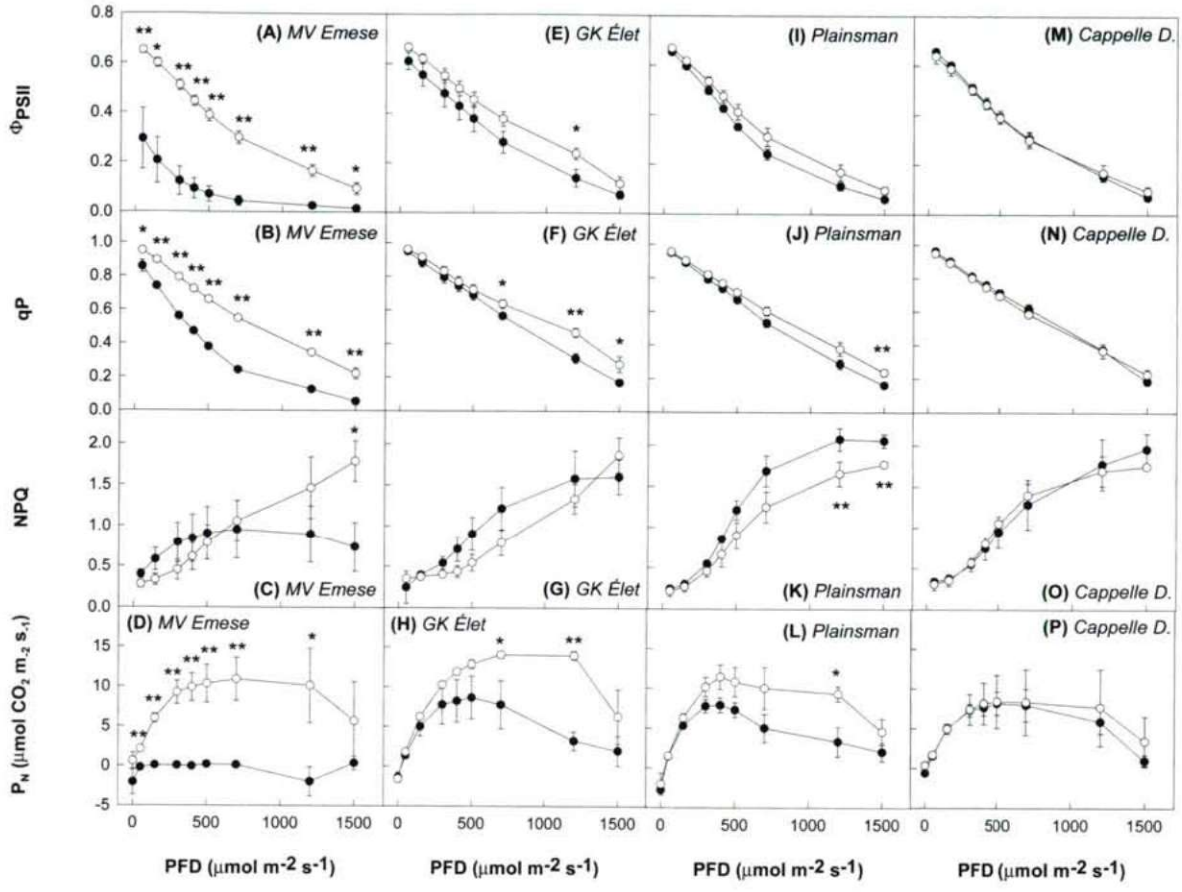


Figure 3. Responses of the actual quantum yield of PSII photochemistry (Φ_{PSII}), photochemical quenching parameter (qP) and non-photochemical quenching (NPQ) to increasing photon flux densities (PFD) in well-watered (o) and water-stressed (●) flag leaves during grain filling in MV Emese (A, B, C), GK Élet (D, E, F), Plainsman (G, H, I) and Cappelle Desprez (J, K, L) wheat cultivars. Data are means ± SD of 3-5 independent samples. SD bars are not shown where smaller than symbols. *, **, *** are for significant differences comparing to the control at 0.05, 0.01, 0.001 levels, respectively.

al. 1996), the variation in leaf area and duration of flag leaf photosynthesis proved also to be important factors under stress conditions (Richards 2000). However, selection for higher rates of leaf photosynthesis has not generally resulted in improved yield under temperate conditions (Evans 1993). Selection for higher photosynthetic rate of flag leaves in early generations improved the grain weight but decreased the total yield (t ha^{-1}) of $F_{5:7}$ imbred lines of wheat (Gutiérrez-Rodríguez et al. 2000).

The yield parameters of the sensitive GK Élet and Cappelle-Desprez declined much more than those of the tolerant MV Emese and Plainsman genotypes. For instance, both the numbers of grains per ear and the thousand-grain dry mass of GK Élet and Cappelle-Desprez decreased to about half of its control and resulted in shrivelled caryopses, while cvs MV Emese and Plainsman showed a mere 21% and 4% decrease, respectively, with apparently filled grains.

The water potential of one tolerant and two sensitive genotypes changed significantly under water deficit, but GK Élet and Cappelle Desprez reached lower Ψ_w, indicating that these plants were physiologically more sensitive. In Plainsman, however, drought affected the plant water status less significantly because water potential was also reduced from 15 DPA in the controls (Guóth et al. 2009). Because rates of aging of leaves accounted for the major differences in carbon fixation per leaf among wheat cultivars (Rawson et al. 1983), the decrease of chlorophyll content shows the rate of flag leaf senescence in control plants and under soil drought. Loss of chlorophyll may be even beneficial in the later stages of grain filling because earlier senescence may indicate an effective mobilization of stored assimilates to the grain (Blum 1998).

The decline in Chl content as a visible symptom of flag leaf senescence could be detected only in sensitive cultivars, Élet and Cappelle upto 12 DPA, and the leaf ZR content decreased significantly only in cv. Élet. Because citokinins maintain the chlorophyll content of tissues and act as antisenescence agents, the decline in ZR in flag leaf of cv. Élet may be in close correlation with an earlier loss of Chls.

Table 2. Effect of soil drought on the final number of grains per ear, mass of grains per ear and thousand-grain dry mass of MV Emese, GK Élet, Plainsman and Cappelle Desprez wheat cultivars. Data are means of the yield parameters in 2007 and 2008 years. Numbers in brackets indicate the percentage of decrease compared to control. *, **, *** are for significant differences comparing to the control at 0.05, 0.01, 0.001 levels, respectively.

	DPA	Chlorophyll *a* well-watered	drought stress	Chlorophyll *b* well-watered	drought stress
MV Emese	0	15.18 ± 1.6	15.60 ± 1.4	5.88 ± 0.8	6.78 ± 0.7
	4	16.06 ± 0.6	15.74 ± 0.6	6.09 ± 0.5	6.21 ± 0.1
	9	15.30 ± 3.4	12.44 ± 2.7	5.84 ± 1.3	5.11 ± 1.2
	12	13.74 ± 1.1	13.41 ± 1.4	3.62 ± 0.9	3.62 ± 0.5
	24	15.11 ± 0.9	5.96 ± 2.2 ***	3.09 ± 1.7	1.46 ± 0.5 **
GK Élet	0	14.18 ± 0.6	12.94 ± 0.4	4.90 ± 0.3	5.02 ± 0.3
	4	11.76 ± 1.2	11.49 ± 2.8	4.26 ± 0.5	4.81 ± 1.1
	9	12.75 ± 1.4	9.62 ± 0.5 *	5.04 ± 0.3	4.76 ± 1.1
	12	13.78 ± 1.7	11.98 ± 0.6	5.13 ± 0.5	4.80 ± 0.3
	24	13.91 ± 0.7	4.19 ± 1.5 ***	6.12 ± 0.7	4.61 ± 2.0 ***
Plainsman	0	15.50 ± 0.3	13.98 ± 1.9	5.75 ± 0.1	5.43 ± 1.0
	4	16.31 ± 1.2	15.08 ± 0.4	6.01 ± 1.0	6.18 ± 0.5
	9	14.40 ± 0.7	12.49 ± 2.4	5.27 ± 0.2	4.92 ± 0.8
	12	16.45 ± 0.9	13.32 ± 1.9	6.38 ± 0.5	6.16 ± 1.0
	24	11.78 ± 1.8	1.37 ± 0.2 ***	4.64 ± 0.6	0.76 ± 0.2 ***
Capelle Desprez	0	14.64 ± 1.1	12.12 ± 0.8 *	5.27 ± 0.3	4.78 ± 0.4
	4	11.90 ± 0.4	11.42 ± 0.9	4.13 ± 0.0	4.50 ± 0.50
	9	12.60 ± 0.8	11.74 ± 0.5	4.63 ± 0.3	4.85 ± 0.4
	12	13.70 ± 0.3	10.08 ± 0.9 *	5.11 ± 0.2	4.01 ± 0.3 **
	24	8.64 ± 0.5	3.02 ± 0.4 ***	4.82 ± 0.5	1.54 ± 0.4 ***

We have assessed chlorophyll *a* fluorescence induction parameters on 16 DPA during the grain filling period in order to reveal those components which are affected by drought and thus are useful physiological traits to screen the drought sensitivity of genotypes before the visible symptoms of fast senescence in flag leaves.

Compared with control plants, there were no significant differences in the maximal quantum yield of PSII photochemistry, F_v/F_m until the 12 DPA, but later a significant decline could be observed in MV Emese, GK Élet and in Cappelle Desprez under water stress demonstrating an earlier disorganisation of PSII reaction centers in water-stressed plants. In cv. Plainsman Fv/Fm tended to decrease in control plants to 24 DPA indicating that the changes were not exclusively induced by drought stress. With the exception of Cappelle Desprez, the A_{max} of light response curves decreased severely in all genotypes under drought that is the biomass production was limited by the stress. The effective quantum yield, Φ_{PSII} and photochemical quenching parameter, qP decreased significantly as a function of PFD in the drought tolerant Emese and in the sensitive GK Élet. Increases in NPQ due to soil drought could only be observed at low PFDs in cv. Emese. The significant decreases in P_N, Φ_{PSII} and qP under drought stress in cv Emese can be explained by the fast response of this cultivar to insufficient irrigation, and the sensitivity of this genotype to high light intensities. Although chlorophyll contents of sensitive genotypes, cvs Élet and Cappelle, were lower and declined from 0-9 DPA under water stress, the rate of chlorophyll loss was much higher in cv. Emese after 12 DPA under drought (Guóth et al. 2009). In the tolerant Plainsman the control plants also showed the symptoms of faster senescence, but Φ_{PSII}, qP and NPQ were slightly affected by WS. In spite the small but significant changes in Chl contents, there were no significant differences in Chl *a* fluorescence induction parameters in the sensitive cv. Cappelle Desprez at 16 DPA. Summarizing the results, neither changes of CO_2 fixation rate nor those of chlorophyll *a* fluorescence parameters in this experiment did not reveal common tendencies which could characterize either tolerant or sensitive genotypes.

Plant senescence induced by water deficit during post-anthesis can promote the remobilization of the pre-stored assimilates to the grains (Kobata et al. 1992; Palta et al. 1994; Zhang et al. 1998; Yang and Zhang 2006). In MV Emese and Plainsman the senescence could result in faster and better remobilization of the pre-stored carbon from vegetative issues, and an ultimately better yield than in GK Élet and Cappelle Desprez under drought. In spite of the sustained CO_2 fixation activity of the flag leaves, the limited utilization of stem reserves in the sensitive cultivars GK Élet and Cappelle Desprez led to severe shrivelling of the grains, and thus to a loss in 1000-grain mass under water stress. Lower citokinin transport from roots to leaves and possibly to developing grains in cv.

GK Élet may contribute to reduced grain number per ear in case of this cultivar.

Although chlorophyll fluorescence is considered a useful tool for screening wheat cultivars under dry conditions (Flagella et al. 1995), its combination with other methods may provide a more accurate assessment of drought tolerance.

Acknowledgements

The authors gratefully acknowledge the financial support of the National Office for Research and Technology of the Republic of Hungary (Grant "Búzakalász", Grant No. NKFP 4/064/2004 and Grant OTKA, Grant NO. T46692, Interreg and Grant "Teller Ede" Grant No. 2006ALAP3-01435/2206.).

References

Bernier G (1998) The control of floral evocation and morphogenesis. Ann Rev Plant Physiol Plant Mol Biol 39:175-219.

Bilger W, Björkman ÖB (1990) Role of xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in *Hedera canariensis*. Photosynthesis Res 25:173-185.

Bilger W, Schreiber U (1986) Energy-dependent quenching of dark level chlorophyll fluorescence in intact leaves. Photosynt Res 10:303-308

Blum A (1998) Improving wheat grain filling under stress by stem reserve mobilisation. Euphytica 100:77-83.

Davies WJ, Metcalf J, Lodge TA, de Costa AR (1986) Plant growth substances and the regulation of growth under drought. Aust J Plant Physiol 13:105-125.

Dodd IC, Stikic R, Davies WJ (1996) Chemical regulation of gas exchange and growth of plants in drying soil in the field. J Exp Bot 47:1457-1490.

Erdei L, Jensén P, Bérczi A, Bengtsson B, Kylin A (1986) Effects of switches in nutrient levels during the life cycle of winter wheat. Physiol Plant 66:583-588.

Evans LT (1993) Processes, genes and yield potential. In Buxton DR, Shibles R, Forsberg RA, Blad BL, Asay KH, Paulsen GM, Wilson RF eds., International Crop Science, I. Madison: Crop Science Society of America, Inc,. pp. 687-696.

Fangmeier A, Brockerhoff U, Grüters U, Jäger HJ (1994) Growth and yield responses of spring wheat (*Triticum aestivum* L. cv. Turbo) grown in open-top chambers to ozone and water stress. Environ Pollut 83:317-325.

Flagella Z, Pastore D, Campanile RG, Di Fonzo N (1995) The quantum yield of photosynthetic electron transport evaluated by chlorophyll fluorescence as an indicator of drought tolerance in durum wheat. J Agric Sci Cambidge 125:325-329.

Flexas J, Escalona JM, Evain S, Gulías J, Moya I, Osmond CB, Medrano H (2002) Steady-state chlorophyll fluorescence (Fs) measurements as a tool to follow variations of net CO_2 assimilation and stomatal conductance during water-stress in C_3 plants. Physiol Plant 114:231-240.

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990:87-92.

Fracheboud Y, Leipner J (2003) The application of chlorophyll fluorescence to study light, temperature, and drought stress. In DeEll JR, Toivonen PMA eds., Practical Applications of Chlorophyll Fluorescence in Plant Biology, Kluwer Academic Publishers, Dordrecht, pp. 125-150.

Gallé Á, Csiszár J, Secenji M, Guóth A, Cseuz L, Tari I, Györgyey J, Erdei L (2009) Glutathione transferase activity and expression pattern during grain filling in flag leaves of wheat genotypes differing in drought tolerance: response to water deficit. J Plant Physiol (accepted).

Guóth A, Tari I, Gallé Á, Csiszár J, Pécsváradi A, Cseuz L, Erdei L (2009) Comparison of the drought stress responses of tolerant and sensitive wheat cultivars during grain filling: changes in flag leaf photosynthetic activity, ABA levels and grain yield. J Plant Growth Reg 28(2):167.

Gutiérrez-Rodríguez M, Reynolds MP, Larqué-Saavedra A (2000) Photosynthesis of wheat in a warm, irrigated environment.II: Traits associated with genetic gains in yield. Field Crop Research 66:51-62.

Hassan IA (2006) Effects of water stress and high temperature on gas exhchange and chlorophyll fluorescence in *Triticum aestivum* L. Photosynthetica 44:312-315.

Hura T, Grzesiak S, Hura K, Thiemt E, Tokarz K, Wedzony M (2007) Physiological and biochemical tools in drought-tolerance detection in genotypes of winter triticale: accumulation of ferulic acid correlates with drought tolerance. Ann Bot 100:767-775.

Kobata T, Palta JA, Turner NC (1992) Rate of development of postanthesis water deficits and grain filling of spring wheat. Crop Sci 32:1238-1242.

Li R-H, Guo P-G, Baum M, Grando S, Ceccarelli S (2006) Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. Agr Sci China 5:751-757.

Lichtenthaler HK, Wellburn AR (1983) Determination of carotenoids and chlorophyll *a* and *b* of leaf extracts in different solvents. Biochem Soc Trans 11:591-592.

Lu C, Zhang J (1999) Effects of water stress on photosystem II photochemistry and its thermostability in wheat plants. J Exp Bot 50:1199-1206.

Paknejad F, Nasri M, Moghadam HRT, Zahedi H, Alahmadi MJ (2007) Effects of drought stress on chlorophyll fluorescence parameters, chlorophyll content and grain yield of wheat cultivars. J Biol Sci 7:841-847.

Palta JA, Kobata T, Turner NC, Fillery IR (1994) Remobilization of carbon and nitrogen in wheat as influenced by post-anthesis water deficits. Crop Sci 34:118-124.

Rawson HM, Hindmarsch JH, Fischer RA, Stockman YM (1983) Changes in leaf photosynthesis with plant ontogeny and relationships with yield per ear in wheat cultivars and 120 progeny. Austr J Plant Physiol 10:503-514.

Richards RA (1988) A tiller inhibition gene in wheat and its effect on plant growth. Austr J Agric Res 39:749-757.

Subrahmanyam D, Subash N, Haris A, Sikka AK (2006) Influence of water stress on leaf photosynthetic characteristics in wheat cultivars differing in their susceptibility to drought. Photosynthetica 44:125-129.

Tari I, Camen D, Coradini G, Csiszár J, Fediuc E, Gémes K, Lazar A, Madosa E, Mihacea S, Poor P, Postelnicu S, Staicu M, Szepesi Á, Nedelea G, Erdei L (2008) Changes in chlorophyll fluorescence parameters and oxidative stress responses of bush bean genotypes for selecting contrasting acclimation strategies under water stress. Acta Biol Hung 59:335-345.

Ugarte C, Calderini CF, Slafer GA (2007) Grain weight and grain number responsiveness to pre-anthesis temperature in wheat, barley and triticale. Field Crops Res 100:240-248.

van den Boogaard, R, Veneklaas, EJ, Lambers, H (1996) The association of biomass allocation with growth and water use efficiency of two *Triticum aestivum* cultivars. Aust J Plant Physiol 23:751-761.

Weiler EW, Jourdans PS, Conrad W (1981) Levels of indole-3-acetic acid in intact and decapitated coleoptiles as determined by a specific and highly sensitive solid-phase enzyme immunoassay. Planta 153:561-571.

Yang J, Zhang J (2005) Grain filling of cereals under soil drying. New Phytol 169:223-236.

Yang JC, Zhang JH, Wang ZQ, Zhu QS, Liu LJ (2003) Involvement of abscisic acid and cytokinins in the senescence and remobilization of carbon reserves in wheat subjected to water stress during grain filling. Plant Cell Environ 26:1621-1631.

Zhang J, Sui X, Li B, Su B, Li J, Zhou D (1998) An improved water-use efficiency for winter wheat grown under reduced irrigation. Field Crops Res 59:91-98.

Zlatev ZS, Yordanov IT (2004) Effects of soil drought on photosynthesis and chlorophyll fluorescence in bean plants. Bulg J Plant Physiol 30:3-18.

Volume 53(1):9-15, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

PCR based isolation and cloning of HMW glutenin gene(s) from wheat (*T. aestivum* var. PBW343) and its fusion with kafirin gene promoter of sorghum

Avinash Mishra[1]*, Sangita Bansal[1], Akash Tomar[1], V. K. Khanna[2] and G. K. Garg[1]

[1]Department of Molecular Biology and Genetic Engineering, G. B. Pant University of Agriculture and Technology, Pantnagar-263145, Uttarakhand, India, [2]Department of Genetics and Plant Breeding, G. B. Pant University of Agriculture and Technology, Pantnagar- 263145, Uttarakhand, India

ABSTRACT With the aim of grain sorghum improvement to enhance its economic value, HMW glutenin gene subunit(s) were isolated from wheat (*T. aestivum* var. PBW343) using allele specific primers (AS-PCR) designed on the basis of allelic data available in NCBI Gene Bank. Three glutenin genes *viz.* Ax1, Dx5 and Dy10 were isolated, cloned, sequenced and analyzed using on line bioinformatics tools. These sequences showed significant homology with the HMW glutenin gene sequences available in the NCBI gene bank. To facilitate transformation of these genes to sorghum, these genes were transferred to a pUC based vector under the regulation of kafirin promoter of sorghum which makes it specific for expression in the endosperm of sorghum. HMW glutenin gene cloned in pGEM vector was excised using restriction enzymes SacI and SacII and directional cloning was performed successfully to clone under the control of kafirin gene promoter of sorghum. Transfer of these gene(s) could be useful for improving the bread making quality of sorghum.

Acta Biol Szeged 53(1):9-15 (2009)

KEY WORDS

cloning
gene cassette
glutenin
kafirin
promoter
sorghum
wheat

Sorghum (*Sorghum bicolor* L.) is a major food crop in the semi-arid tropics of Asia and Africa. In these areas where the people are poor and food resources are limited, sorghum is a major source of protein. Traditionally, in the developing countries, sorghum is consumed as porridge or flat bread. It contains similar levels of starch and protein as in other cereals and hence is considered nutritious. However, some factors like poor dough making quality, low digestibility and presence of tannin make it less palatable. Although sorghum is widely used and consumed but poor shelf life and inability to make soft, fluffy, palatable and easily digestible roti or bread limits its role in the economic empowerment of sorghum producers and end users.

For the regular use of sorghum it is important that it must have good bread making quality as that of wheat. Recombinant DNA technology has tremendous potential to enhance the bread making quality of sorghum grain by transferring the bread making character of bread wheat into sorghum. After a very long investigation, it was found that the glu D1 encoded HMW glutenin subunit pair 5+10 and glu 1Ax1 is associated with greater dough strength of the wheat (Shewry and Halford, 2002). Ovidio and Anderson (1994) confirmed the role of glu D1 (Dx5- Dy10) in bread making quality of wheat. They analyzed that the y- type subunits are the main components responsible for dough making quality of the flour while x-type subunit have only minor effect.

Altpeter et al. (2004) generated and characterized transgenic rye synthesizing substantial amounts of high-molecular-weight glutenin subunits (HMW-GS) from wheat. They reported that the amount of polymerized glutelins was significantly increased in transgenic rye, more than triple as compared to the wild type. The expression of wheat HMW-GS in rye leads to a high degree of polymerization of transgenic and native storage proteins, probably by formation of intermolecular disulfide bonds. This is an important step towards improving bread-making properties of economically important grains.

HMW-GS significantly contributes towards dough improvement and for these, glutenin alleles of wheat are important genetic resources for quality improvement. For the better use and quality improvement, it is important to use the indigenous sources of the genes. Indian resource of the genes is well acclimatized in Indian condition and there will be zero risk of the gene flow, as the genes are well established. Exotic genes may disturb our gene pools emerging and also by drifting native genes. Biosafety regime also encouraged the use of indigenous resources. Indian cultivated wheat var. PBW 343 was a good choice for the glutenin genes. Compared to other wheat cultivars, it has Dx5-10 and 1Ax1, glu gene alleles. With the aim of improvement of grain sorghum to enhance its marginal value for poor consumers and producers, the pres-

Accepted June 10, 2009
*Corresponding author. E-mail: avinash@csmcri.org

Figure 1. Amplification of HMW glu gene(s), using allele specific primer. Lane-ML : Marker 100 bp ladder, Lane- 1 : glu Dy10 (3 band app. Size 2.0, 1.8 & 1.0 KB), Lane-2 & 5: glu Dx5 (band app. Size 2.0 kb), Lane-3 & 4: glu 1Ax1 (band app. Size 1.8 kb), Lane- M : Marker Lambda DNA dd Hind III/ EcoR I.

ent study was done, in which HMW glutenin genes, 1Dx5, 1Dy10 and 1Ax1 were amplified using PCR with designed gene specific primers and fused with the kafirin gene promoter of sorghum which is previously isolated and characterized (Mishra et al. 2008).

Material and Methods

Isolation of genomic DNA

Seeds of wheat (*Triricum aestivum*) variety PBW-343 were procured from Crop Research Center G.B. Pant University, Pantnagar, India. The variety was selected as it shares alleles x1(Glu-A1), x5+y10 (Glu-D1), x7+y9 (Glu-B1). One gram of etiolated leaves were taken and weighed. These were cut into small pieces and then frozen in liquid nitrogen. The frozen leaves were grinded into fine powder in a pre- cooled mortar with the help of a chilled pestle. Powder was transferred into 50 ml centrifuge tube (Oakridge tube, Labware, USA) containing 15 ml DNA extraction buffer (0.1 M Tris, 0.05 M EDTA, NaCl, pH 8.0) and 1 ml of 20% SDS. After proper mixing, the tube was incubated in a water bath (Hoefer, USA) at 65°C for 10 minutes. 10 ml of 5 M Potassium acetate was added and mixed the content of the tube gently and further incubated on ice for 20 minutes. Tube was centrifuged at 10,000 rpm for 25 min at 4°C in Kendro Biofuge refrigerated centrifuge. Supernatant was collected in a fresh oakridge tube and 15 ml isopropanol was added and mixed gently. The tube was incubated at -20°C for 1 hr and then centrifuged at 10,000 rpm at 4°C for 15 min to collect the DNA pellet. Gently poured off the supernatant and dried the pellet. Pellet was dissolved in 1 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNase treatment was given to genomic DNA as RNase stock (10 mg/ ml) was added to give final concentration of 10µg/ ml and incubated for 1 hr at 37°C. The DNA was extracted with phenol, chloroform and isoamyl alcohol (25:24:1). To the supernatant, collected in a fresh tube, 0.1 volume 3 M sodium acetate (pH 4.8) was added followed by 0.6 volume cold isopropanol. DNA was precipitated by keeping the tube

Table 1. Restriction digestion analysis of the pGEMT-HMW plasmids.

Details	Restriction enzymes		Expected No. of bands and band size		Observed No. of bands and band size	
	Enzymes used	Enzymes which have restriction sites	No. of bands	Band size	No. of bands	Band size
HMW glutenin gene Dx5	SmaI,ClaI, DraI, XmaI, SmaI, XbaI, SacI, SacII, SalI, EcoRI, EcoRV, BamHI, BglI, KpnI, PstI, NotI,SphI, SpoI	HindIII	2	500 bp 1.5 kb	2	500 bp 1.5 kb
		BglI	2	163 bp 1.8 kb	2	100p 1.8 kb
		SphI	2	254 bp 1.7 kb	2	200p 1.7 kb
HMW Glutenin gene Dy10	— Do —	Bam HI	2	129 bp 1.3 kb	2	100 bp 1.3 kb
		Hind III	2	265 bp 1.2 b	2	200 bp 1.2 kb
HMW Glutenin gene Ax1	— Do —	SphI	2	134 bp 1.7 kb	2	100 bp 1.6 kb
		SpoI	2	205 bp 1.6 kb	2	200 bp 1.6 kb
		HindIII	2	320 bp 1.5 kb	2	300 bp 1.5 kb

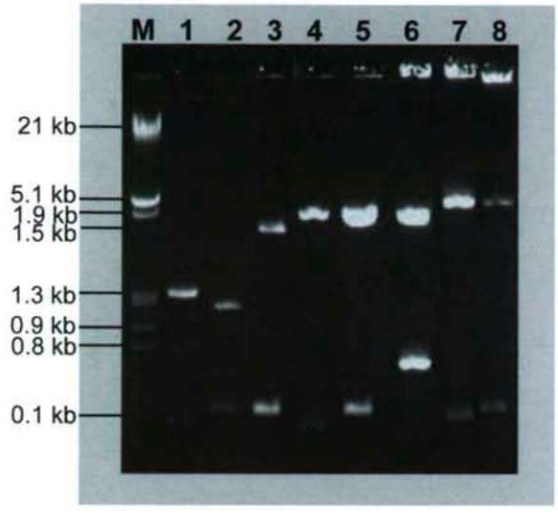


Figure 2. Restriction digestion of putative HMW glutenin gene(s) clones. Lane- M: Marker, Lane- 1: RD by Bam HI of glu Dy10 (2 band app. Size 0.1 & 1.3 kb), Lane- 2: RD by Hind III of glu Dy10 (2 band app. Size 0.2 & 1.2 kb), Lane- 3: RD by Hind III of glu 1Ax1 (2 band app. Size 0.3 & 1.5 kb), Lane- 4: RD by Sph I of glu 1Ax1 (2 band app. Size 0.1 & 1.6 kb), Lane- 5: RD by Spe I of glu 1Ax1 (2 band app. Size 0.2 & 1.6 kb), Lane- 6 : RD by Hind III of glu Dx5 (2 band app. Size 0.5 & 1.5 kb) Lane- 7 : RD by Bgl I of glu Dx5 (2 band app. Size 0.15 & 1.7 kb) Lane- 8: RD by Sph I of glu Dx5 (2 band app. Size 0.25 & 1.7 kb).

Figure 3. PCR of putative clones using allele specific primer. Lane- 1: PCR on gDNA, glu Dy10 (3 band app. Size 2.0, 1.8 & 1.0 KB), Lane-2: PCR on gDNA, glu Dx5 (band app. Size 2.0 kb), Lane-3 & 4: PCR on gDNA, glu 1Ax1 (band app. Size 1.8 kb), Lane- M: Marker Lambda DNA dd Hind III/ EcoR I, Lane- 5: PCR on putative plasmid, glu Dx5 (band size 2.0 kb), Lane- 6: PCR on putative plasmid, glu 1Ax1 (band size 1.8 kb), Lane- 7: PCR on putative plasmid, glu Dy10 (band size 1.4 kb), (g DNA- genomic DNA).

at -20°C for overnight and then pelleted by centrifuging at 10,000 rpm at 4°C for 12 min. DNA pellet was dried after washing with 70% ethanol and re-dissolved in 100 µl of TE buffer and then stored at -20°C.

PCR based amplification of HMW glutenin gene(s)

Allele specific primers (F: 5' – GAC AGT CCA CCG AGA TGG - 3', R: 5' – GCA AGC TGC AGA GAG TTC - 3' for Dy10; F: 5' – CAT GGT CCT GAA CCT TCA CC - 3', R: 5' – CAG AGA GTT CTA TCA CTG GC - 3' for Dx5 and F: 5' – CCG AGA TGA CTA AGC GG - 3', R: 5' – GCT AA CAT GGT ATG GGC T - 3' for 1Ax1) were designed for the isolation of HMW- glu gene(s) allele on the basis of HMW glutenin gene sequence available on NCBI data bank (gene accession no. X13928, X12928 and X12929 for the HMW glutenin gene Ax2, Dx5, Dy10 respectively). A master mix (25 µl) was prepared, containing 40 ng of the template DNA, 100 ng of each primer (F & R), 200 µM of each dNTPs, 1 x assay buffer and 2.0 unit of Taq DNA polymerase in a 0.2 ml of sterile, thin walled PCR tubes (Axygen). PCR was carried out in Biometra thermal cycler with initial denaturation temperature of 94°C for 5 min, subsequent 35 cycles of 94°C denaturation for 1 min, 54°C annealing for 2 min and 2 min of the extension at 72°C. Final extension was done at 72°C for 5 min. During PCR, lid temperature of the cycler was fixed at 104°C (to prevent evaporation). After the completion of PCR cycles, a sub sample (usually 10 µl) of the amplicons was analyzed on 1.8% agarose gel by electrophoresis and remaining were stored at -20°C for subsequent experiments.

Cloning of PCR amplicons

PCR amplified putative HMW glutenin gene(s) were purified using MinElute PCR Purification System (Qiagen, Germany), following manufacturer's instructions. An aliquot of the purified putative glutenin gene(s) was analyzed on agarose gel before using it in the ligation reaction to confirm its elution from the MinElute column (Qiagen, Germany). For the ligation, pGEM-T Easy vector (Promega, USA) and insert DNA were briefly centrifuged to collect contents at the bottom of the tube. A Ligation reaction (10 µl), containing 1x ligase buffer (300 mM Tris pH 7.8, 100 mM $MgCl_2$, 100 mM DDT, 5 mM ATP), 1 µl (50 ng) pGEM vector, 1 µl (3 unit) T4 DNA ligase and appropriate amount of HMW glutenin gene (insert 75 ng for Dy10, 100 ng for Dx5 and 90 ng for 1Ax1), was set up in 0.5 ml sterile tube. Ligation mixture was mixed gently and then incubated overnight at 4°C in a refrigerated water bath (Hoefer, USA). Ligation mix (5 µl) was transformed to competent cells (100 µl) of *E. coli* strain DH 5α. All putative positive clones were selected and confirmed by PCR using gene specific primers, universal T7 & SP6 primers and restriction digestion. Reselected clones were sequenced by automated DNA sequencer. Sequences of the clones were subjected for the blast and studied using online available bioinformatics tools and submitted to NCBI (www.ncbi.nlm.nih.gov) gene data bank.

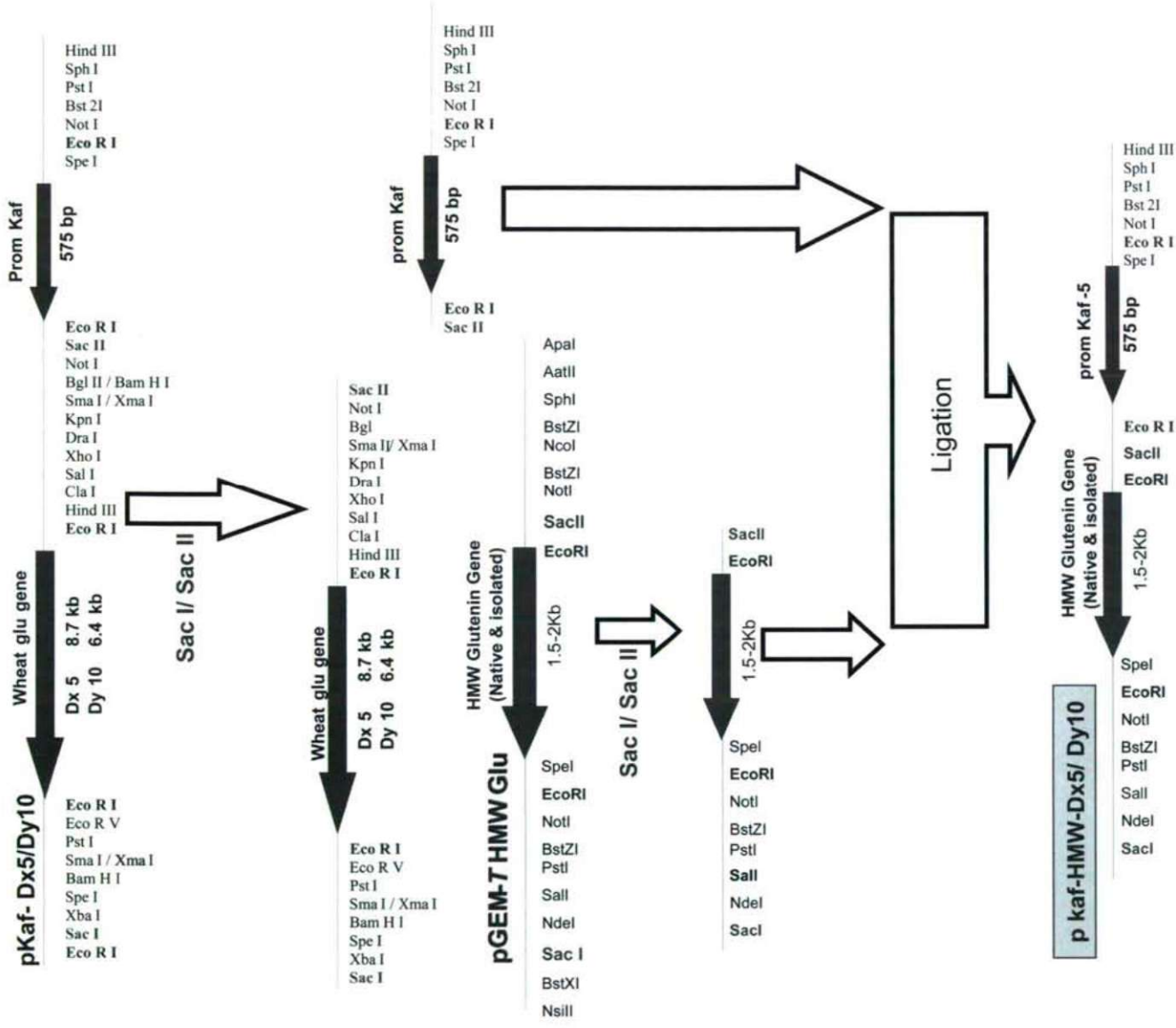


Figure 4. Strategy for construction of gene cassette pKaf-HMW-Dx5/ Dy10 (Indigenous HMW glu gene is driven by gamma kafirin promoter of sorghum).

Construction of a gene cassette (HMW glutenin gene regulated by Kafirin promoter

Restriction digestions were performed by incubating gene cassette pKaf-glu (previously constructed, Mishra et al. 2008b) and pGEM-HMW (HMW glutenin gene cloned in pGEM) with restriction enzymes SacII and SacI. A measure of 20 µl reaction mixture containing 15 µl plasmid DNA sample, 1 unit of enzyme, 2 µl of the respective digestion buffer and 1 x BSA (if needed) was prepared for restriction digestion in a 0.5 ml sterile tube. Tube was incubated at 37°C for overnight in circulating water bath. Restriction digestion was analyzed on 1.2% agarose gel by electrophoresis. Digested indigenous HMW glutenin gene and vector pkaf was gel eluted using same MinElute PCR Purification System (Qiagen, Germany). Vector without gene and indigenous HMW gluten genes both were ready to ligate. Ligation reaction (15 µl) containing, 1 µl Vector(Kafirin promoter vector), 6 µl Insert (Indigenous HMW glutenin gene), 5 unit T4 DNA Ligase and 1 x ligation buffer, was set up and tube incubated at 4°C for 12 hr, then subsequently at 8°C, 16°C and finally 22°C for 1 hr each. 5 µl of each ligated mixture was transformed aseptically in 100 µl E. *coli* DH 5α competent cells. Recombinants were selected

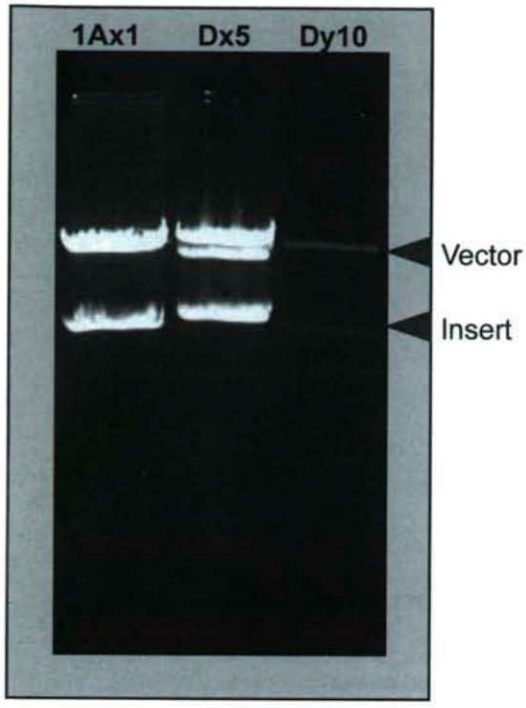


Figure 5. Elution of *glu* gene(s) from vector pGEM-T easy.

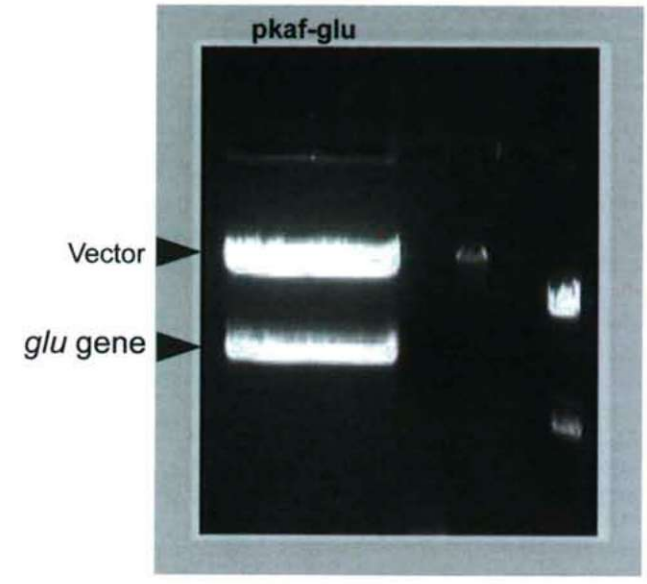


Figure 6. Elution of pkafΔglu (pKaf without *glu* gene).

and analysis of gene construct was done by restriction digestion. Recombinants were named as kaf-HMW (kaf-HMW-A, kaf-HMW-Dx and kaf-HMW-Dy for the gene cassette harboring HMW glutenin gene Ax1, Dx5 and Dy10, respectively with Kafirin promoter).

Result and Discussion

HMW gene amplification and cloning

Sun et al. (2004) developed allele-specific (AS) PCR to isolate and clone HMW-GS genes from cultivated emmer. They showed that single band of strong amplification was obtained through AS-PCR of genomic DNA from emmer. In present study, HMW glutenin gene subunits were amplified using de-

Table 2. Restriction digestion analysis of gene cassette pkaf-HMW-glu (HMW glutenin gene driven by γ- kafirin promoter of sorghum).

Clones	Restriction Enzyme	Expected Results		Observed Results (app.)	
		No. of Bands	Band Size	No. of Bands	Band Size
pkaf-HMW-1Ax (5475 bp)	EcoRI	4	3000 bp 1800 bp 575 bp 100 bp	4	3000 bp 1800 bp 500 bp 100 bp
	PstI	2	3000 bp 2475 bp		3000 bp 2400 bp
pkaf-HMW- Dx 5 (5675 bp)	EcoRI	4	3000 bp 2000 bp 575 bp 100 bp	4	3000 bp 2000 bp 500 bp 100 bp
	PstI	2	3000 bp 2675 bp	2	3000 bp 2500 bp
pkaf-HMW- Dy 10 (5145 bp)	EcoRI	4	3000 bp 1470 bp 575 bp 100 bp	4	3000 bp 1400 bp 500 bp 100 bp
	PstI	2	3000 bp 2145 bp	2	3000 bp 2000 bp

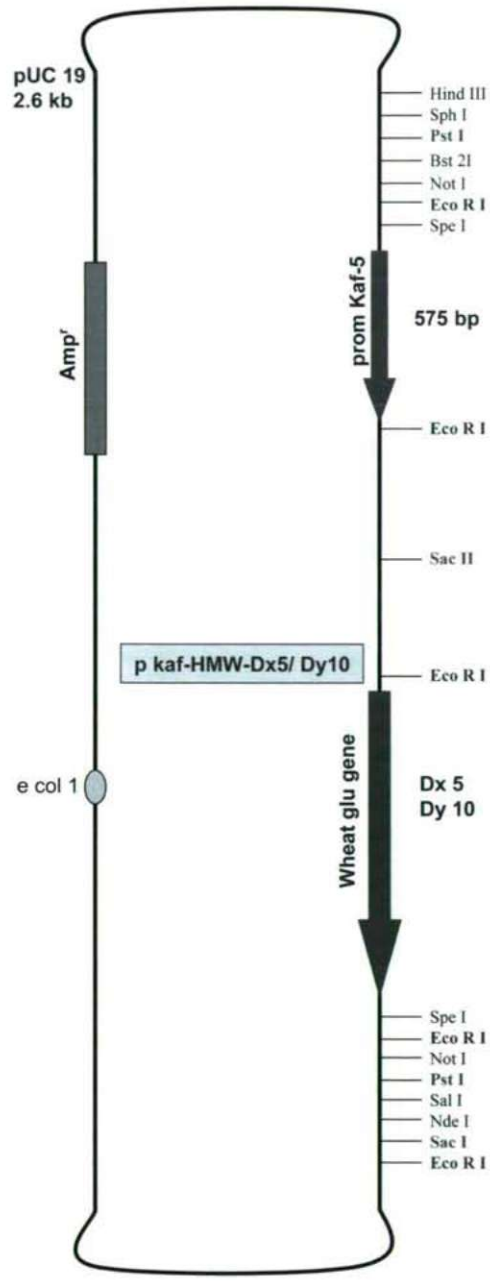


Figure 7. Restriction map of gene construct pKaf-HMW-glu gene(s).

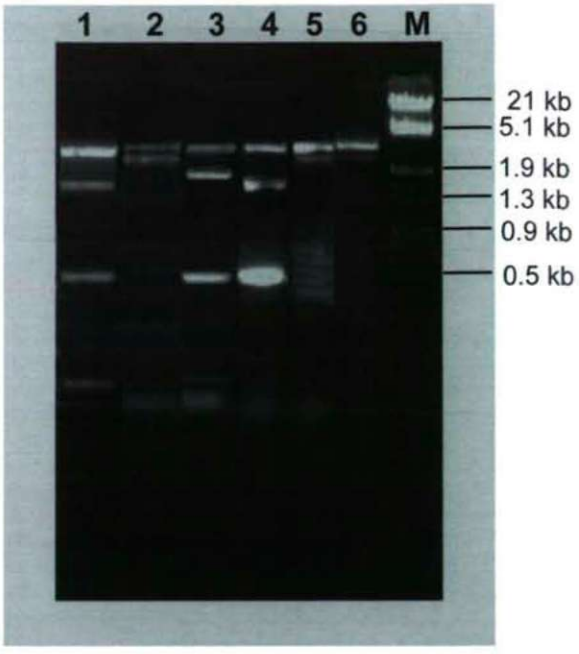


Figure 8. Restriction digestion of putative recombinants pKaf-HMW-glu (Indigenous glu gene driven by kafirin promoter). Lane- 1: RD by Eco RI of glu 1Ax1 (4 band app. Size 3.0, 1.8, 0.5 & 0.1 kb), Lane- 2: RD by Pst I of glu 1Ax1 (2 band app. Size 3.0 & 2.4 kb), Lane- 3 : RD by Eco RI of glu Dx5 (4 band app. Size 3.0, 2.0, 0.5 & 1 kb), Lane- 4: RD by Eco RI of glu Dy10 (4 band app. Size 3.0, 1.4, 0.5 & 0.1 kb), Lane- 5: RD by Pst I of glu Dy10 (2 band app. Size 3.0 & 2.0 kb), Lane- 6: RD by Pst I of glu Dx5 (2 band app. Size 3.0 & 2.5 kb), Lane- M: Molecular wt. marker.

signed gene specific primers. Three amplicons of approx. size 2.0, 1.4 & 1.0 kb were amplified with glu Dy10 primers while one intact band of 2.0 kb and 1.8 kb was found with Dx5 and 1Ax1 glutenin primers, respectively (Fig. 1). Multiple bands in Dy10 could be due to two probable reasons- first is that the primers designed for Dy10 subunits from NCBI data may not be highly specific for Dy10 of PBW 343. Therefore they could fatuously amplify some other HMW glutenin subunit like 'By' subunit from the wheat genomic DNA. Second is that due to chance the primers could anneal anywhere in the genome and amplify some other regions. PCR amplicons contain hanging 'A' (because it has been amplified by Taq DNA polymerase), so vector to be used should contain hanging 'T'. Different cloning vectors are available for the cloning of PCR products. pGEM T- easy vector system (Promega, USA) was used to clone the PCR amplicons (putative HMW glutenin genes). According to their designed primer pair, the expected size of the amplicon was 1.5 kb for the HMW glutenin subunits Dy10 therefore amplicon of size 1.5 kb was eluted from the agarose gel, quantified and cloned. Besides this Dx5 and 1Ax1 were also cloned in same vector. Putative recombinant plasmids (p-GEM-HMW) were isolated from all white colonies and electrophoresed on 1% agarose gel. The amplified PCR products were cloned at the multiple cloning sites (MCS) hence each clone will have a molecular size of approximately 5 kb. The presence of insert was confirmed by a series of restriction digestion analysis (Table-1, Fig. 2) and PCR using gene specific primers (Fig. 3). The selected recombinants for 1Ax1, Dx5 and Dy10 of size 1.8 kb, 2.0 kb and 1.47 kb were sequenced and submitted to NCBI (accession no.DQ211817, DQ211818 and DQ211819, respectively). All the sequences were searched for homology on blast tool of

the NCBI web site. All the clones showed 97-99% homology with HMW glutenin gene of *Triticum aestivum.*

Construction of a gene cassette (Kafirin promoter with indigenous HMW glutenin gene)

The main objective of the research is to develop transgenic sorghum having good bread making quality. For the expression of HMW glutenin gene, it must be driven by γ- kafirin gene promoter of sorghum. Since the glutenin gene(s) are to be transformed to sorghum, pkaf –Dx5/ Dy10, a vector engineered by us and is carrying kafirin promoter (specific for driving expression in endosperm, Mishra et al. 2008a) was selected. A gene cassette was constructed (Fig. 7) by directional cloning of HMW glutenin gene (Fig. 4) isolated HMW glutenin gene of wheat variety PBW 343 in a gene cassette pkaf- Dx5/ Dy10 (Mishra et al. 2008b) constructed earlier.

Sub cloning of indigenous HMW glutenin gene

Plasmid pGEM-HMW and gene construct pkaf- Dx5/ Dy10 (procured HMW glu gene driven by Kafirin promoter) were isolated and subjected for restriction digestion with SacII and SacI, resultant HMW glutenin gene from pGEM as well as from vector pkaf- Dx5/ Dy10 were excised. Isolated indigenous HMW glutenin genes and pkaf glu (kafirin promoter without glutenin gene) were eluted from the gel (Fig. 5 and 6, respectively) and purified. The HMW glutenin gene was cloned downstream to the kafirin gene promoter as Figure 4. Subcloned gene(s) were transformed to competent cells of *E. coli* strain DH 5 α and recombinants were selected on the selection marker ampicillin (50µg/ ml). All colonies were probable recombinants as there is no chance of self ligation (both ends are different hence non-compatible). Recombinants were named as kaf-HMW (kaf-HMW-A, kaf-HMW-Dx, kaf-HMW-Dy for the gene cassette having HMW glutenin gene Ax1, Dx5, Dy10 respectively with Kafirin promoter). Thus by directional subcloning expression vectors pkaf-HMW-Dx5 and pkaf-HMW- Dy10 were constructed (Fig. 7). Plasmid DNA of recombinants (pkaf-HMW-glu) was isolated in bulk and purified and checked by 0.8% agarose gel electrophoresis. Proper cloning was confirmed by restriction digestion with EcoRI and PstI (Fig. 8). The results of the restriction digestion was summarized in Table 2. The recombinants gave the result as per expectation and support the proper directional cloning.

Conclusion

In the present study, it has been possible to clone all the three glutenin genes *viz.* Dx5, Dy10 and Ax1 from wheat (*T. aestivum* var. PBW 343) and significant sequence homology was observed. Wheat variety PBW 343 is known to be good for bread making because of the qualities of its glutenin products. Hence transfer of these gene(s) could be useful in improving the bread making quality of sorghum to facilitate transformation of these genes to sorghum, these genes have been transferred to a pUC based vector under the regulation of kafirin promoter of sorghum which makes it specific for expression in the endosperm of sorghum.

References

Altpeter F, Popelka JC, Wieser H (2004) Stable expression of 1Dx5 and 1Dy10 high molecular weight glutenin subunit genes in transgenic rye drastically increases the polymeric glutelin fraction in rye flour. Plant Mol Biol 54:783-792.

Mishra A, Tomar A, Bansal S, Khanna VK, Garg GK (2008a) Temporal and spatial expression analysis of *gamma* kafirin promoter from Sorghum *(Sorghum bicolor* L. moench) var. M 35-1. Mol Bio Rep 35:81-88.

Mishra A, Pandey R, Bansal S, Tomar A, Khanna VK, Garg, GK (2008b) Construction of gene cassette harboring HMW glutenin gene of wheat driven by γ-kafirin promoter of sorghum. Acta Biologica Szegediensis 52:277-282.

Ovidio DR, Anderson OD (1994) PCR analysis to distinguish between alleles of a member of a multigene family correlated with wheat bread making quality. Theor Appl Genet 88:759-763.

Pandey R, Mishra A, Garg GK (2008) Plant promoter driven heterologous expression of HMW glutenin gene(s) subunit in *E. coli*. Mol Bio Rep 35:153-162.

Shewry PR, Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 53:947-958.

Sun M, Yan Y, Jiang Y, Xiao Y, Hu Y, Cai M, Li Y, Hsam SL, Zeller FJ (2004) Molecular cloning and comparative analysis of a y-type inactive HMW glutenin subunit gene from cultivated emmer wheat (*Triticum dicoccum* L.). Hereditas 141:46-54.

Volume 53(1):17-20, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Relationship between virus traffic and nitric oxide (NO) production in tobacco roots

Oana Danci[1,#], Zsuzsanna Kolbert[2,#*], Marcel Danci[1], Bernadett Bartha[2] and László Erdei[2]

[1]Department of Plant Biotechnology, Faculty of Horticulture, 'Banat's University of Agricultural Sciences, Timisoara, Romania, [2]Department of Plant Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

ABSTRACT Nitric oxide (NO) was proved to have several roles in plant-pathogen interactions from the contribution to the local and systemic induction of defence genes to the infection signalling. The aim of this paper was to prove the involvement of NO as signalling molecule during virus infection and to point out that determination of NO levels can be used as a new method of virus detection in plants. For detection of NO generation in tobacco plants infected by potato viruses X, Y and A, 4,5 diaminofluorescein-diacetate (DAF-2DA) was used. It was found that the infected tobacco roots showed two to three times higher NO accumulation, compared to control. Our results indicated that long distance virus movement through roots occured in the stele using phloem sieve elements, but most of the viruses did not reach meristem and root cap cells. It is suggested that NO is a proper signalling molecule during virus infection and fluorescent detection of NO makes possible to demonstrate the presence of viruses within the plant tissues.

Acta Biol Szeged 53(1):17-20 (2009)

KEY WORDS

nitric oxide
potato virus X
potato virus Y
potato virus A
virus trafficking

Nitric oxide is a highly reactive molecule that rapidly diffuses and permeates cell membranes. This novel bioactive molecule is a free radical that can either gain or lose an electron to reach energetically more favourable structures, namely nitrosonium cation (NO^+) and nitroxyl anion (NO^-). Because of its unique chemistry, which permits both its stability and reactivity, NO and its exchangeable redox-activate forms are now recognized as intra- and intercellular signalling molecules (Wilson et al. 2008). During the last few years NO has been detected in several plant species and the increasing number of reports on its function in plants have implicated NO as a key molecular signal, that participates in the regulation of several stress responses (Erdei and Kolbert 2008 and references therein). NO has been also implicated in disease resistance to virulent and avirulent pathogen attack (Delledonne et al. 1998). In particular, NO has a significant role in plant resistance to pathogens by contributing to the local and systemic induction of defence genes (Romero-Puertas et al. 2004; Wendehenne et al. 2004; Hong et al. 2008). Most of the experimental data available on NO detection during plant-pathogen interactions come from studies on infections by biotrophic pathogens (Romero-Puertas et al. 2004; Foissner et al. 2000), but there are no reports available on NO production in roots as pathogen stress response.

There are two basic routes by which a virus can move through the plant to give a full systemic infection: cell-to-cell movement through plasmodesmata and long distance movement (Scholthof 2005). Cell-to-cell or short distance movement means, that the virus spreads from the initially infected cell, which is usually epidermal or mesophyll cell to the vascular bundle and during long distance movement the virus travels then through the vascular tissue, usually the phloem sieve-tubes. As known from literature, the virus needs three days to pass into the vascular system and the first part infected is the root followed by the youngest leaves of the plant (Hull 2001).

According to our best knowledge, there are no reports reflecting on development of virus infection-induced NO generation in roots. Since after infection in the leaf, viruses appear first in roots before returning to the shoot system, our aim was to follow the NO production in time and in location in virus- infected primary roots. Therefore we carried out *in vivo* and *in situ* determination of NO levels in tobacco roots infected by potato virus X, Y and A using fluorescence microscopic method.

Materials and methods

Plant material and growth conditions

Six-weeks-old *Nicotiana tabacum* L. SR1 plants (Medgyesy et al. 1980) were transferred from soil to modified Hoagland nutrient solution for another week and were grown under controlled conditions in greenhouse at photo flux density of 240 $\mu mol\ m^{-2}\ s^{-1}$ (16/8 h day/night period) at relative humidity of 55-60%, and 25 ± 2°C temperature. After a week, plants were mechanically inoculated at the second leaf counted from the

Accepted June 8, 2009
*Corresponding author. E-mail: kolzsu@bio.u-szeged.hu
#These authors contributed equally to this work.

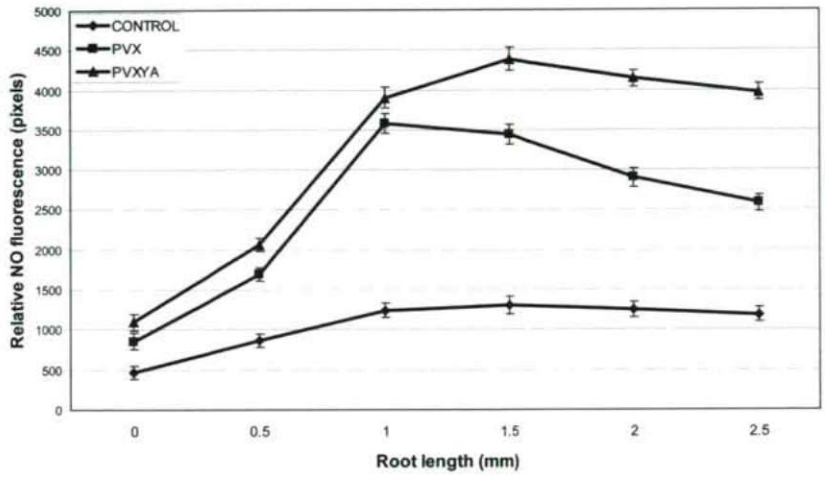


Figure 1. Localization of NO fluorescence along the length of primary roots of Nicotiana tabaccum L. SR1 plants three days after inoculation through the leaves with Potato virus X (PVX, ■), or a mixture of three viruses Potato virus X, Y and A (PVXYA, ▲). Untreated plants served as control (♦). NO levels were determined as described in Materials and Methods. Vertical bars are standard errors (n= 3-5).

basis with *Potato virus X* or with a mixture of three viruses: *Potato virus X, Potato virus Y* and *Potato virus A* and three days later NO fluorescence was detected in roots. Healthy, non-infected plants constituted the control.

Detection of NO

Visualization of NO was performed according to the highly sensitive *in situ* and *in vivo* method of Kojima et al. (1998) with some modifications. About 2.5 cm long root segments were cut and dyed with 10 μM 4,5-diaminofluorescein-diacetate (DAF-2DA) for 20 minutes at 25 ± 2°C in darkness. Cross- sections were cut with a sharp razor. After dyeing, the samples were washed 4 times within 20 minutes with 2-(N-morpholino) ethanesulfonic acid (MES) buffer (10^{-3}M, pH 6.15). To detect fluorescence intensity, Zeiss Axiowert 200M-type fluorescent microscope (Carl Zeiss, Germany) equipped with filter set 10 (exc.: 450-490 nm, em.: 515-565 nm) was used. To measure the fluorescence intensity, Axiovision Rel. 4.5 software was applied. The same camera settings were recorded for each digital image.

Detection of NO fluorescence was performed at days 3, 5, 7, 9, 11 and 13 after the infection of plants at their second leaf counted from the basis. In order to understand the virus traffic within the plant, measurements were done every 0.5 mm from the root tip to 2.5 mm distance on a 0.4 mm diameter circle. In each treatment, at least 3 samples were measured. When time-dependence of NO intensity was followed, measurements were done at the 2.5 mm of the root as from the tip.

Results and Discussion

The long-distance movement of viruses after mechanical infection of the leaves is immediately directed to the roots where it turns back towards the shoot apex and afterwards the infection spreads to the whole shoot system (Hull 2001). Therefore, in our experiments, first the expected changes in the supposedly concomitant NO accumulation were localized in space and time. In roots, intensity of NO levels varied basipetally, showing maxima from 1 to 2 mm distance from the tip, depending on treatments (Fig. 1). The lowest NO levels were seen within the first 0.5 mm of the root tip, which includes root cap and meristem and the maximal values, were detected between 1 and 2 mm from the tip. After 2 or even 2.5 mm from the tip NO fluorescence intensity slowly decreased or reached a constant value. Comparing the results of infected samples with control ones it was observed that infected roots showed at least two times higher level of NO than the control (Fig. 1). Thus, it was concluded that under our experimental conditions an increased NO production was involved in the response reaction to virus infection (Gould et al. 2003) or as a plant defence mechanism against the pathogens (Delledone et al. 1998). Other reports showed that root tips of plants infected with one of several viruses have been found to be free of detectable virus (Appiano and D'Agostino 1983). Smith and Schlegel (1964) studied the distribution of *Clover yellow mosaic virus* in root tip of *Vicia faba* and found that within the limit of the assay method, the first 0.4 mm of the root tip (root cap and meristem) was virus free. According to these results in our experiments, NO production in virus infected root tip regions was not higher than that in the root tips of the healthy control plants (Fig. 2 abc), leading to the conclusion that the majority of the viruses are not entering the meristematic cells (Hull, 2001). But, in the cases of mixed infections (PVX, PVY and PVA) some of the viruses were trafficking to the tip of the root infecting also the meristematic cells. This process was accompanied by NO accumulation

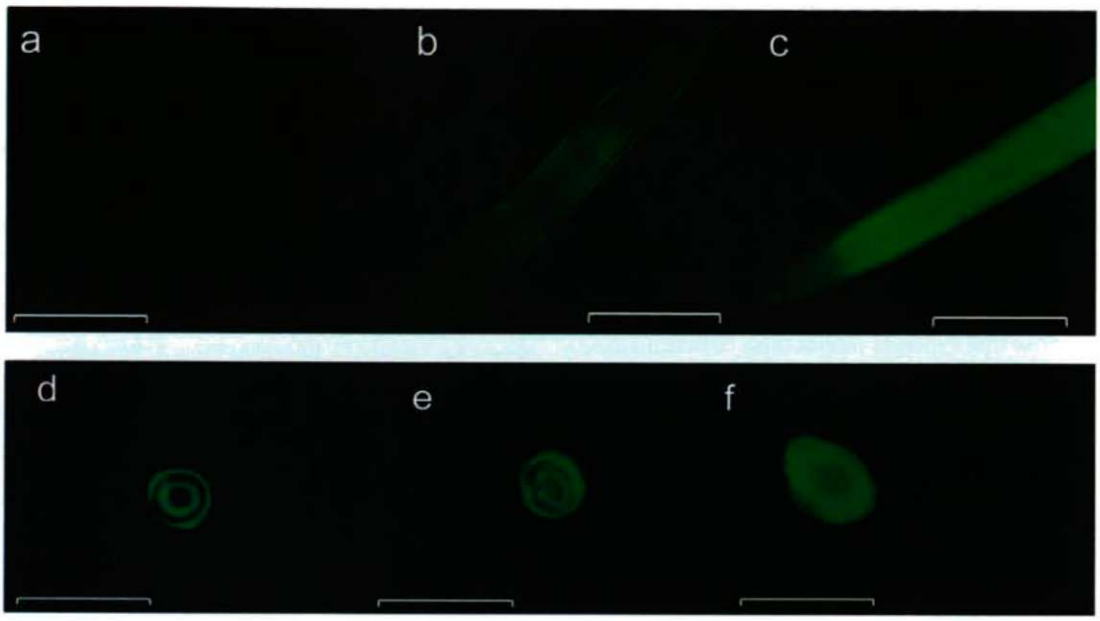


Figure 2. Fluorescence microscopic visualization of nitric oxide in the roots: a) control roots of healthy tobacco; b) PVX- infected root; c) PVX, PVY and PVA- infected root. Representative photographs of 831 pictures. Visualization of NO by fluorescence microscopy in root cross- sections: d) control roots of healthy tobacco; e) PVX- infected root; f) PVX, PVY and PVA- infected root. Representative photographs of 78 pictures. Samples were prepared as described in Materials and Methods. Bars= 1 mm.

(Fig. 1), since NO-related fluorescence intensity was higher in PVXYA- infected root tips than in those of control roots. This provides evidence that some viruses and also in mixed infections may invade the primary meristematic tissues. In order to localize the tissue used for virus transport, fine cross sections were cut from the root using a sharp razor. It was found that in control samples NO fluorescence was restricted only to the cortex cells, but in infected roots stele also showed intensive NO fluorescence (Fig. 2 def). These results suggest that the virus transport occured through the vascular bundle, which is in agreement with other literature data (Hull 2001).

NO fluorescence was determined in the 3rd, 5th, 7th, 9th, 11th and 13th day after the mechanical virus inoculation and it was found that with a single exception (7th day), the NO formation

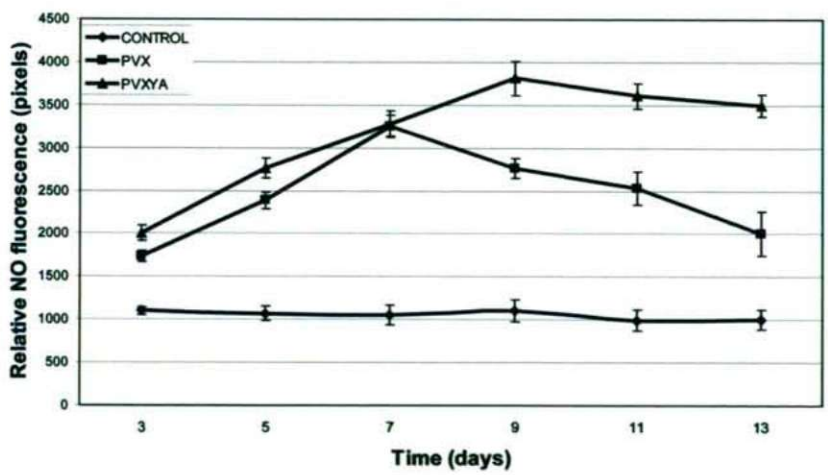


Figure 3. Time dependence of viral infection evolution in primary roots of Nicotiana tabacum L. SR1 plants after three days of inoculation their leaves with Potato virus X (PVX, ■), or a mixture of three viruses Potato virus X, Y and A (PVXYA, ▲). Untreated plants served as control (♦). NO levels were determined as described in Materials and Methods. Vertical bars are standard errors (n= 3-5).

was the highest in roots infected by the virus mixture (PVX, PVY and PVA), followed by those infected with PVX alone, but all showed higher levels of NO than the control. These are in accordance with other results showing that a plant sensibility increases with multiple infections (Faccioli and Zoffoli 1998). In the cases of PVX- infected roots NO fluorescence reached its maximum value on the 7th day, while in samples infected by virus mixture the highest NO level was detected later, on the 9th day after infection. This phenomenon can be due to the difference between their transport rates within the plant. We suppose that synergic interactions between viruses might result in lower speed of the viral complex in the mixed infection. The decrease of NO fluorescence after the seventh or the ninth day, respectively, may be due either to the development of the plant defence mechanisms against virus infection (Hull 2001) or to the feedback of high concentration of NO, which might be toxic for the pathogen (Mur et al. 2003). Curves of NO fluorescent values showed a minimum and a maximum, depending on the virus, while NO levels in control roots prooved to be constant during the whole experimental period (Fig. 3).

According to these results we conclude that NO is a proper signalling molecule during virus infections and investigation of NO-coupled fluorescence can be used as a method of virus detection in plants.

Acknowledgements

Virus material was provided by Dr. Nicolae Cojocaru, Potato Research Institute, Brasov, Romania. This study was carried out while O Danci and M Danci stayed at the Department of Plant Biology, University of Szeged, in the framework of the Hungarian-Romanian PHARE Cross-Border Cooperation Project to L Erdei (project No. HU 2003/005.830.01-04).

References

Appiano A, D'Agostino G (1983) Distribution of tomato bushy stunt virus in root tips of systemically infected Gomphrena globosa. J Ultra Res 85:239-248.

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394:585-588.

Faccioli G, Zoffoli R (1998) Fast eradication of potatovirus X (PVX) and potato virus S (PVS) from virus-infected potato stem-cuttings by chemotherapy. Phytopathol Medit 37:9-12.

Foissner I, Wendehenne D, Langebartels C, Durner J (2000) In vivo imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J 23:817-824.

Gould KS, Lamotte O, Klinger A, Pugin A, Wendehenne D (2003) Nitric oxide production in tobacco leaf cells: a generalized stress response? Plant Cell Environ 26:1851-1862.

Hong JK, Yun B-W, Kang J-G, Raja MU, Kwon E, Sorhagen K, Chu C, Wang Y, Loake GJ (2008) Nitric oxide function and signalling in plant disease resistance. J Exp Bot 59:147-154.

Hull R (2001) Matthew's Plant Virology, Academic Press, Fourth Edition, San Diego CA. 373-411.

Kojima H, Nakatsubo N, Kikuchi K, Urano Y, Higutchi T (1998) Direct evidence of NO production in rat hippocampus and cortex using a new fluorescent indicator: DAF-2DA. Neuroreport 9:3345-3348

Erdei L, Kolbert Zs (2008) Nitric oxide is a potent signalling molecule in plants. Acta Biol Szeged 52:1-5.

Medgyesy P, Menczel L, Maliga P (1980) The use of cytoplasmic streptomycin resistance: Chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvestris*, and Isolation of their somatic hybrids. Mol Gen Genetics 179:693-698.

Mur LAJ, Carver TLW, Prats E (2006) NO way to live; the various roles of nitric oxide in plant-pathogen interactions. J Exp Bot 57:489-505.

Romero-Puertas MC, Perazzolli M, Zago ED, Deledonne M (2004) Nitric oxide signalling in plant-pathogen interactions. Cell Microbiol 6:795-803.

Scholthof HB (2005) Plant virus transport: motions of functional equivalence. Trends Plant Sci 10:376-382.

Smith SH, Schlegel DE (1964) The distribution of Clover yellow mosaic virus in *Vicia faba* root tips. Phytopathol 54:1273-1274.

Wendehenne D, Durner J, Klessing DF (2004) Nitric oxide: a new player in plant signalling and defence responses. Curr Opin Plant Biol 7:449-455.

Wilson ID, Neill SJ, Hancock JT (2008) Nitric oxide synthesis and signalling in plants. Plant Cell Environ 31:622-631.

Volume 53(1):21-26, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Physiological and anatomical comparison between four different apple cultivars under cold-storage conditions

Saleh A. M. Ghafir[1], Suliman O. Gadalla[1], Benissa N. Murajei[1] and Mohamed Fathi El-Nady[2]*

[1]Department of Horticulture, Faculty of Agriculture, Omar AlMukhtar University, EL-Beida, Libya, [2]Department of Agricultural Botany, Faculty of Agriculture, Kafrelsheikh University, Egypt

ABSTRACT The work was carried out in two successive seasons to investigate the storability of four apple cultivars viz. Golden Delicious, Starking Delicious, Star Cremson and Gala, which were grown under El Jabal El Akhdaer conditions, in Libya. Fruits were harvested and stored at 85-90% relative humidity and 0°C for 0, 30, 60, 90, 120, 150 and 180 days. The obtained results exhibited the significant differences between the tested cultivars in the studied parameters comprising physiological and anatomical parameters. The fruits of Star Cremson cv. had a potentially good storage properties, as they showed less weight loss, while those of Gala cv. had the highest level of weight loss. On the other hand, Golden Delicious cv. gave the highest values of fruit firmness, while the lowest values were obtained in fruits of Starking Delicious and Star Cremson cvs. fruits. Fruit weight loss % increased, while other studied parameters (firmness rate, TSS%, starch concentration and acidity%) decreased gradually by extending storage period. Anatomical data demonstrated that fruit surface of cvs. Gala and Star Cremson were smooth, while undulate and ripples surfaces were obtained in Starking Delicious and Golden Delicious cvs., respectively at the end of cold storage. Gala fruits had the thick cuticle layer compared to the other cultivars. Crushed parenchymatous cells (cpc) were found in storage tissue in Starking Delicious and Golden Delicious. It may be concluded that the results indicated a great variability among cultivars. All studied parameters of four apple cultivars reduced during cold-storage periods. The highest fruit storability was achieved for Star Cremson.
Acta Biol Szeged 53(1):21-26 (2009)

KEY WORDS

Apple cultivars
cold-storage
cuticle
crushed parenchymatous cells
storability

Apple (*Malus domestica* L.) is a member of family *Rosaceae* and distributed worldwide. The growth period and development of fruit is not only important as a basic problem in plant physiology, but it is also of economic importance during transportation and storage (Ben-Arie and Lurie 1986). During this period, different biochemical and physical changes take place associated with changes in different morphological characters (Hulme 1971). Several observations reveal relationship between fruit characters and temperature. This is an important point, because of the frequent use of low temperature during the post-harvest storage of fruit. One of the roles of temperature is its regulatory effect on the rate of chemical reactions, resulting in a slowing of the rate of metabolism when the temperature falls. The level of phenolic compounds can be affected by cold as a result of slowing of natural ripening (Macheix et al. 1990).

Fruit skin in apples consists of the cuticle, epidermis and several layers of hypodermis (Babos et al. 1984). Both skin and its waxy coat are important during transportation and storage. Its mechanical resistance is directly related to apple fruit damage (Pierzynowska-Kornik et al. 2002). The skin thickness is not uniform in apples, it differs considerably between cultivars and between different years within the same cultivar, (Homutova 2005 and Homutova and Blazek 2006). Skin characteristics influence fruit bruising to a large extent. The skin thickness influences the penetration of pathogens into fruits (Mourichon and Bompeix 1979). Also, the apple skin structure and its thickness clearly affect the storage ability of fruits (Ruffa et al. 1992). The thickness of the epidermis and the surface wax are the main skin characteristics that are related to weight losses during cold-storage (Bebic 1972 and Veravrbeke et. al. 2003).Therefore, the aim of this study was to quantify the physiological and anatomical differences in apples between selected cultivars under cold storage conditions.

Materials and Methods

This work was carried out in two successive seasons (2005 and 2006) at the Horticultural Department., Faculty of Agriculture, Omar Al Mukhtar University, in Libya and the Department of Agriculture and Botany, Faculty of Agriculture, Kafrelsheikh University in Egypt. Four grown apple cultivars:

Accepted June 12, 2009
*Corresponding author. E-mail: mfmelnady@yahoo.com

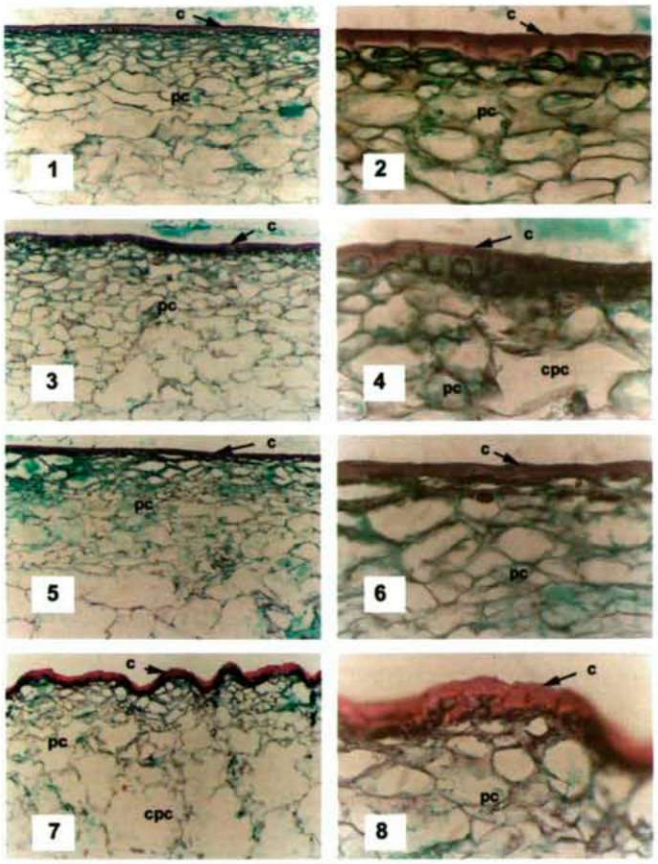


Figure 1. Transfer-sections in surface parts of apple cultivars fruits. 1-2: Gala 3-4: Starking Delicious, 5-6: Star Cremson, 7-8: Golden Delicious. Cuticle (c), parenchyma cells (pc), crushed parenchyma cells (cpc). X 100 (1,3,5 and 7), X 400 (2, 4, 6 and 8).

Golden Delicious, Starking Delicious, Star Cremson and Gala under El Jabal El Akhdaer conditions, in Libya, were used in the experiments. The location is characterized by an average annual temperature of 13.1°C, average rainfall about 600 mm and altitude about 750 m above sea level. Fruits were harvested at commercial maturity from trees grafted on MM 106 rootstock and chosen as well as were stored in controlled chamber at 85-90% relative humidity and 0°C for 0, 30, 60, 90, 120, 150 and 180 days. Experiment was designed as split plot design with four replicates, where main plots assigned for cultivars and subplots for storage periods. Differences between the tested apple cultivars by the use of physical (fruit weight loss % and fruit firmness (FF) %) and chemical (TSS%, starch concentration and acidity) as well as anatomical parameters of fruits stored under cold conditions were investigated monthly.

Physiological parameters

The physiological parameters were measured to the subsequent criteria:

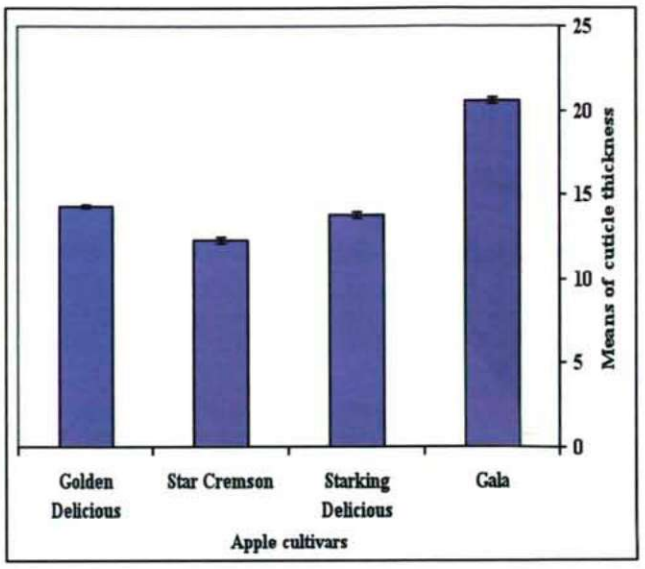


Figure 2. Means of cuticle thickness (µm) of fruit of four apple cultivars under cold storage conditions.

Fruit weight loss (FWL) %

Ten fruits of each treatment were selected and assigned with code number for each fruit and each of them was weighed before and after the storage period and then weight loss percentage was calculated as follows:

$FWL\ \% = FW_1\text{-}FW_2/FW_1 \times 100$

Where FW_1 is fruit weight before cold storage and FW_2 is fruit weight after cold storage periods.

Fruit firmness rate (kg/cm^2): Fruit firmness was measured by Ham-held magness-pressure tester of (5-15) inch plunger, according to A.O.A.C (1981).

Chemical parameters

Total soluble solid (TSS)%: was determined by the use of a hand refractometer (Carl-Zeiss).

Titratable acidity: values were measured by malic acid estimation in apple fruits according to Rangana (1979).

Starch concentrations percentage values: were measured by using iodine staining (2.5 g iodine + 10 g potassium iodide in 1 L distilled water) according to Teskey and Shoemaker (1978).

Anatomical parameters

For preparing transfer-sections, the fruit specimens were taken 90 days after cold storage from the surface parts (about 1/2 cm^3) at the maximum fruit diameter of different studied cultivars. Specimens were fixed in Formalin Alcohol Acetic Acid mixture (FAA, 1:18:1 v/v), washed and dehydrated in alcohol series. The dehydrated specimens were infiltrated and embedded in paraffin wax (52-54°C m.p.). The embed-

Table 1. Differences between the tested apple cultivars by using some physical and chemical parameters under storage-cold conditions.

Apple varieties	Weight loss %		Firmness rate kg/ cm²		T.S.S %		Starch concent.		Acidity %	
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
Golden Delicious	6.89b	2.80b	2.51c	2.48c	13.07b	14.07b	3.50	3.53	0.16c	0.15c
Starking Delicious	6.12c	2.47c	3.16a	3.19a	13.57ab	14.57ab	3.50	3.57	0.19a	0.19a
Star Cremson	5.63d	2.42c	3.14a	3.23a	13.46ab	14.46ab	3.39	3.39	0.17b	0.17b
Gala	8.7a	4.13a	2.74b	2.85b	13.94a	14.91a	3.46	3.46	0.18a	0.19a
F test	**	**	**	**	**	**	NS	NS	**	**

Mean values within a column followed by different letter are significantly different at P≤0.5 level.

Table 2. Effect of storage periods on some physical and chemical characters of cold-stored fruits.

Storage periods	Weight loss %		firmness rate kg/ cm²		T.S.S %		Starch concent.		Acidity %	
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
0 day	0.00f	0.00g	4.47a	3.63a	14.25a	15.25a	5.18a	5.21a	0.23a	0.24a
30 day	6.05e	2.26f	2.44d	2.75d	13.87b	14.88b	5.00a	5.06a	0.22a	0.22b
60 day	7.16d	2.71e	3.57a	2.89c	13.68c	14.69bc	3.68b	3.75b	0.21b	0.21c
90 day	8.06c	3.12d	2.64c	2.81c	13.56bc	14.56cd	3.25c	3.43b	0.19c	0.19c
120 day	8.38d	3.61c	3.00b	2.74d	13.34cd	14.34de	3.25c	2.93c	0.16d	0.16e
150 day	9.23a	4.23b	273c	3.11b	13.09de	14.09ef	3.06d	2.37d	0.12e	0.11f
180 day	9.03a	4.77a	2.44d	2.63e	12.78e	13.72f	1.68e	1.68e	0.11f	0.10g
F test	**	**	**	**	**	**	**	**	**	**

Mean values within a column followed by different letter are significantly different at P≤0.5 level.

ded specimens were sectioned on a rotary microtome at a thickness of 10-12 µm. Sections were mounted on slides and deparaffinised. Staining was accomplished with safranine and light green, cleared in xylol and mounted in Canada balsam, according to Ruzin, 1999. Slides were microscopically examined and measurements and counts were taken and averages of 10 readings from 3 slides were calculated.

Statistical analyses

The obtained data were subjected to the proper statistical procedures for analysis of variance according to that outlined by Gomez and Gomez (1984) using Genstat software as an analytical tool.

Results and Discussion

Physical and Chemical Parameters

Effect of cultivars

Fruit weight loss % showed that, there were significant differences between four apple cultivars in the present study. Star Cremson had retained good quality after a long-term cold storage, as they showed less fruit weight loss percentage while cv. Gala had a low storability during all cold storage periods (Table 1). It is clear that, fruit storability correlated with fruit firmness rate (FFR) and anatomical analysis (Figures 1 and 2 and Table 4). The thickness of main skin, cuticle and surface wax characteristics are related to weight loss percentage in cold-stored apples (Bebic 1972 and Ruffa et al. 1992). Several previous works indicated there was a correlation between different cultivars and fruit weight losses which were stored under cold conditions (Guleryuz et al. 2000; Bandaravicius et al. 2001; Ugolik et al. 2001; Tavares et al. 2002; Baneh et al. 2003).

Starking Delicious and Star Cremson had the highest fruit firmness values in both seasons while the lowest values were recorded in Golden Delicious. These results are in harmony with the anatomical data (Figs. 1 and 2). FFR values are directly related to cuticle layer thickness. Several observations reveal relationship between Ca content in fruits and FFR values (Zheng et al. 2005; Sasnauskas 2006).

Concerning, total soluble solid (TSS) percentage, no significant differences were obtained between cvs Gala, Star Cremson and Starking Delicious. The highest and the lowest TSS% values were recorded in Gala and Golden Delicious, respectively. Clearly, TSS% associated with fruit storability, it increased gradually by increasing in fruit weight loss %. Saquet and Streif (2000) revealed the relationship between TSS% and ethylene production and fruit ripening in Gala cultivar. These results coincided with those obtained by Naseri et al. (2001); Baneh et al. (2003); Hamedi and Milani (2003); Song et al. (2003) and Ventura and Sansavini (2005).

Table 3. Effect of apple cultivars and storage periods interactions on some physical and chemical characters of fruits stored under cold conditions.

Varieties	Storage periods	Weight loss %		Firmness rate kg/ cm²		T.S.S %		Starch concent.		Acidity %	
		2005	2006	2005	2006	2005	2005	2006	2005	2006	2005
Golden Delicious	0 day	0.00g	0.00g	3.81bc	5.35d-f	13.62	14.62	5.50	5.50	0.28a	0.28
	30 day	6.03mn	1.88p	1.97n	2.75h-k	13.37	14.38	4.75	5.00	0.22cd	0.21d-f
	60 day	6.86kl	2.57m-o	3.11fg	2.63i-k	13.00	14.00	3.75	6.25	0.19fg	0.18h
	90 day	7.97gh	2.66k-m	2.23k-n	2.30lm	13.50	14.50	3.25	3.75	0.16i	0.15i
	120 day	8.56f	3.48g-i	2.47jk	2.14m	13.12	14.12	3.00	3.25	0.12j	0.12jk
	150 day	9.32e	4.37de	2.07l-n	2.08m	12.50	13.50	2.50	3.00	0.08k	0.07l
	180 day	9.50de	4.86c	1.93n	2.12m	12.37	13.38	1.75	2.50	0.05l	0.04m
Starking Delicious	0 day	0.00g	0.00g	3.17e-g	3.89a	14.50	15.50	5.25	1.75	0.21d-f	0.22de
	30 day	5.57po	1.98op	2.82h	3.61b-d	13.87	14.88	5.00	5.25	0.20e-g	0.20f-h
	60 day	6.27m	2.50l-n	3.93ab	3.27ef	14.00	15.00	3.50	5.00	0.24bc	0.23cd
	90 day	7.23j	2.54l-n	3.09fg	2.92h	13.5	14.50	3.25	3.50	0.20d-g	0.20e-h
	120 day	7.12jk	2.94j-l	3.21ef	2.77h-k	13.37	14.38	2.75	3.75	0.16i	0.16i
	150 day	8.29fg	3.39h-j	3.28ef	3.23fg	13.00	14.00	2.75	2.75	0.15i	0.15i
	180 day	8.56f	3.95ef	2.84h	2.63ik	1275	13.75	2.00	2.00	0.16i	0.16i
Star Cremson	0 day	0.00g	0.00g	3.31ef	3.49c-f	14.25	15.25	4.75	4.75	0.20g-e	0.20e-h
	30 day	5.25p	1.69p	2.03mn	3.13m	13.62	14.62	5.00	5.00	0.21g-d	0.21e-g
	60 day	5.83no	2.09n-p	4.12a	3.62a-d	13.50	14.50	3.75	4.00	0.20g-e	0.20f-h
	90 day	6.33m	2.76k-m	2.74hi	3.52b-e	13.37	14.38	3.25	3.50	0.22c-e	0.21d-f
	120 day	6.76l	3.06i-k	3.73de	3.23fg	13.00	14.00	3.25	2.75	0.17hi	0.16i
	150 day	7.61i	3.51f-i	3.30ef	3.63a-c	13.37	14.38	2.25	2.25	0.13j	0.13j
	180 day	7.68hi	3.83f-h	3.14e-g	2.99gh	13.12	14.12	1.50	1.50	0.11j	0.10k
Gala	0 day	0.00g	0.00g	3.61cd	3.79ab	14.62	15.62	5.25	5.25	0.22cd	0.24bc
	30 day	7.35ij	3.47g-i	2.93gh	2.52j-l	14.62	15.62	5.25	5.25	0.26b	0.25b
	60 day	9.67d	3.87fg	3.12fg	2.03m	14.25	15.25	3.75	3.75	0.21d-f	0.21d-f
	90 day	10.72c	4.53b	2.50ij	2.50kl	13.87	14.88	3.25	3.25	0.16gh	0.19h
	120 day	11.08b	4.96cd	2.93gh	2.80hi	13.87	14.88	3.25	3.25	0.19f-h	0.19g-h
	150 day	10.57c	5.63b	2.30j-l	3.51c-e	13.50	14.50	2.00	2.00	0.11j	0.11jk
	180 day	11.69a	6.43a	2.84n	2.78h-i	12.87	13.62	1.50	1.50	0.11j	0.10k
F test		**	**	**	**	NS	NS	NS	NS	**	**

Results of starch concentration measurements, however, showed that no significant differences were recorded between the four apple cultivars in both seasons. It was also observed that significant differences between cultivars in acidity values were found after cold-storage periods. The highest and the lowest acidity were recorded in cvs. Starking Delicious and Golden Delicious, respectively.

Mean values within a column followed by different letter are significantly different at P 0.5 level.

Effect of cold-storage periods

Mean values of fruit weight loss % of four apple cultivars in both seasons are presented in Table (2). Analysis of variance reveals no significant differences between mean values of studied cultivars. Mean values enhanced with the increasing of storage periods. Several observations revealed that relationship between apple fruit weight losses stored under cold conditions and checked. This could be attributed to increase in transpiration, respiration rates and decline in fruit firmness (Blampired, 1981). Similar results were noted by El-Shennawi (1989) on Anna cultivar and Gavalheiro, et al. (2003) on Bravo de Esmolfe and Erturk (2003) on Jonagold, Elesta and Granny Smith cultivars. It can be also noted that, mean values of fruit firmness rate were decreased gradually with extending storage period. The highest values were recorded at beginning and the lowest at the end of storage periods. These results support several previous works Gavalheiro et al. (2003), Erturk et al. (2003), Buchanan and Brock (2005) and Kvikliene et al. (2006).

Concerning TSS% data in Table (2) indicated that, mean values decreased with extending storage period. At the beginning of storage, the highest values were produced, whereas the lowest mean values were at the end. These results may be due to respiration process (Rhodes 1970) and conversion of starch into monosaccharides (Duque et al. 1999). Such results are in harmony with those obtained by Kvikliene et al. (2006). For starch concentration data indicated that, there were significant differences between cultivars. Mean values were reduced with the increasing in fruit ripening and storage period in both seasons. This may be due to starch conversion to simple sugars, El-Shennawi (1989). In addition, acidity values were decreased in four tested apple cultivars with the

Table 4. Means of cuticle thickness of fruit of four apple cultivars stored under cold conditions.

Cultivars	*Means of cuticle thickness (µm)*
Gala	*20. 6a*
Starking Delicious	*13.8b*
Star Cremson	*12.3c*
Golden Delicious	*14.3b*

Mean values within a column followed by different letter are significantly different at P≤0.5 level.

increasing storage periods. This may be due to fruit ripening and respiration processes (Phillps et al. 1954; Knee and Sharples 1981; El-Shennawi 1989; Gavalhero et al. 2003; Kvikliene 2006).

Effect of apple cultivars and storage periods interactions

Data in Table (3) indicated that all studied parameters were reduced in four cultivars and all storage periods during the both seasons. Analysis of variance shows significant differences between mean values of fruit weight loss % and fruit firmness rate values. For fruit weight loss %, cv. Gala gave the highest values at the end of the storage period (180 days). On the other hand, cvs. Gala and Golden Delicious caused the lowest fruit firmness rate. No significant differences were observed in TSS% and starch concentration in both seasons. For acidity %, significant differences were recorded in both seasons. Golden Delicious gave the highest and the lowest values at the beginning and at the end of cold storage, respectively.

Mean values within a column followed by different letter are significantly different at P 0.5 level.

Regarding anatomical structure of the surface of apple fruits after 90 days cold-storage, Figures (1 and 2) and Table 4 illustrate that cvs. Gala and Star Cremson have smooth fruit surface while fruit surfaces in cvs. Starking Delicious and Golden Delicious were undulate and rippled, respectively. Fruit surface shaped related to weight losses percentage. Thickness of cuticle ranged from 12.3 µm in Star Cremson to 20.6 µm in Gala. No significant differences were found between cvs. Starking Delicious and Star Cremson. Crushed parenchymatous cells were found in storage parenchyma tissue in Starking Delicious and Golden Delicious fruits only. The thickness of cuticle and surface wax characteristics are related to weight loss percentages in cold-stored apple (Bebic 1972, and Ruffa et al. 1992). To the authors knowledge it appear that such anatomical study has not been previously investigated. Therefore, this study may be considered the first report on anatomical character of the tested cultivars and a key step for further studies on these cultivars and their susceptibility to postharvest diseases under similar conditions.

From the foregoing results, it may be concluded that, all studied parameters of four apple cultivars were reduced during cold-storage periods. Higher fruit storability was achieved in cv. Star Cremson.

References

A.O.A.C. (1981) Official Method of Analysis Agricultural Chemists11 th Ed. Published by the A. O. A. C., P. O. Box : 540, Washington.

Babos K, Sass P and Mohacsy P (1984) Relationship between the peel structure and storability of apple. Acta Agronomica Academiae Scientiarum-Hungaricae 33:41-50.

Bandaravicius A, Gelvonauskiene D and Sasnauskas A (2001) Investigation of biologically and economically important traits of Introduced apple cultivars. Sodininkyste ir Darzininkyste 20(1):3-15.

Baneh HD, Hassani A, Majidi A, Zomorodi S, Hassani G, Malakooti MJ (2003) Effect of calcium chloride concentration and the number of foliar application on the texture and storge quality of apple (Red Delicious) in Orumia Region. Agricultural Science Tabriz 12 (4):47-54.

Bebic D (1972) Uticaj osobina pokozice ploda jabuke na gubitke u tezini za vreme skladistenja. Zbornik Radova Poljoprivredong Fakulteta 20 (546):1-8.

Ben-Arie R, Lurie S (1986) Prolongation of fruit life after harvest. In: Monselise SP (ed) Handbook of fruit set and development. CRC, Boca Raton, Fla, p. 493.

Blampired GD (1981) A relationship between water loss and storage breakdown of "Mcintosh" apple. HortScience 16(4):525-526.

Duque P, Barreiro MG, Arrabaca JD (1999) Respiratory metabolism dipping cold storage of apple fruit. I-sucrose metabolism and glycolysis. Physiol Plant 107(1):14-23.

EL-Shennawi MRM (1989) Effect of post harvest calcium chloride fruit dipping on quality and storability of "Anna" apple during cold storage. M. Sc. Thesis Alexandria University.

Erturk U, Akbuclak B, Ozer MH (2003) Quality changes of some apple cultivars stored in normal atmosphere for long period. Acta Hortic 599:665-672.

Gavalheiro OJ, Santos A, Recasens I, Larrigancliere C, Silvestre A (2003) Quality of the portuguese "Bravo de Esmolfe" apple after normal cold storage or controlled atmosphere and two shelf life periods. Acta Horticultwe 1:395-400.

Gomez KA, Gomez AA (1984) Statistical procedures for agricultural research. 2nd ed. John Wiley and Sons, pp. 229-308.

Guleryuz M, Ercisli S, Erkan E (2000) Physical and chemical changes during fruit development in apple cultivars in Erzincan plain and their interrelationships. Ziraat Fakultesi Dergisi, Ataturk Universitesi 32 (1):51-59.

Hamedi M, Milani JMZ (2003) Susceptibility of five apple cultivars to browning. Iranian J Agric Sci 34(1):91-96.

Homutova I (2005) Zmêny anatomicke stovby exokarpu plodu u vybranych odrud jabloni. In Zima M, Bolrcek P, Omelka R eds., 4. biologickeprogres v bilogii, 8. -9.9. Nitra, UKF:214-215.

Homutova I and Blazek J (2006). Differences in fruit skin thickness between selected apple (*Malus domestica* Borkh.) cultivars assessed by histological and sensory methods. Hort Sci (Prague) 33(3):108-113.

Hulme AC (1971) The biochemistry of fruits and their products.Vol. 2 Academic Press, London.

Knee M.and Sharples R (1981) The infiuence of controlled atmosphere storage on the ripening of apples in relation to quality. In PW Goodenough and PK Atkins, eds., quality in stored and Processed vegetables and fruit. A Cademic Press London and NewYork. p. 341-352.

Kvikliene N, Kviklys D,Viskelis P (2006) Changes in fruit quality during ripening and storage in the apple cultivar auksis. J Fruit Ornam Plant Res 141:195-202.

Macheix JJ, Fleuriet A and Billot J (1990) Regulation of phenolic metabolism in fruit temperature. In Fruit phenolics. CRC, Boca Raton, Fla, p. 199.

Mourichon Z, Bompeix G (1979) Mise en evidence des voies de penetration du *Phytophthora cactorum* (L. et C.) Schroeter dans les pommes Golden Delicious. Etude realisee en microscopie electronique a balayage. Fruits 34:761-766.

Naseri L, Babalar M, Askary MA, Talaei AR (2001) Effects of foliar sprays of some micronutrients and cold storage on fruit quality of Golden Delicious apple. Iranian J Agric Sci 32(3):591-597.

Phillips WR, Poapst PA, Rheaume BJ (1954) The Effect of temperature near 32 degree F. on storage behavior of "Mcintosh" apple. Amer Soc Hort Sci 65:214-222.

Pierzynowska-kornika G, Zadernowski R, Fornal J (2002) The microstructure of selected apple varieties. Journal of Polish Agricultural Universities, Food Science and Technology, Volume 5, Issue 2, Available Online: http://www.ejpau.media.pl/series/volume5/issue2/food/art-13.html.

Rangana S (1979) Manual of analysis of fruits and vegetables products. Mc - Graw Hill Pub. Company.

Rhodes (1970) The climacteric and ripening of fruit. In AC Hulem ed., The biochemistry of fruit and their products. Vol.1. A Cadmic press, London and New York., pp. 521-533.

Ruffa E, Me G and Dore B (1992). Histochemical study of some apple cultivars pericarp application of the crystabilization technique in ethylene glycol (SO_3). Histochem J 24:549-550.

Ruzin SE (1999) Plant microtechnques and microscopy. First Ed. Oxford University Press, USA.

Saquet AA and Streif J (2000) Investigations on the respiration and the ethylene production of some new apple cultivars. Erwerbsobstbau 42(4):109-112.

Sasnauskas A, Gelvonauskiene D, Duchovskis P, Siksnianien JD, Sabajevi-ene G (2006) Investigation of introduced biological traits of apple tree cultivars. Sodininkyste ir Darzininkyste 25(1):3-12.

Song K, Hwang J, Ahn SY (2003) Seasonal changes of enzyme activity related to sugar metabolism in leaves of apple cultivars differing in maturty. Journal of the Korean Society for Horticultural Science 44(2):211-214. Resources 6(4):67-77.

Tavares JT, Silva CL, Cardoso RL Silva MA, Carvalho LA and Santos CMG (2002) Postharvest application of calcium chloride in fruits of the mountain apple (*Eugenia malaccensis* L). Magistra 14(2):article l.

Teskey BJ and Shoemaker JS (1978) Tree fruit production. AVI Publishing Co. Inc. Westport Connecticut.

Ugolik M, Zydlik Z, Kantorowicz MB and Pacholak E (2001). Yield and fruit quality of selected apple cultivars. Prace z Zakresu Nauk Rolniczych 91:181-186.

Ventura M and Sansavini S (2005) Typology of fruiting branches and quality of apple cultivars Braeburn, Golden Delicious and Jonagold. Rivista di Frutticoltura e di Ortofloricoltura 67(11):52-60.

Veravrbeke EA, Verboven P, Oostveldt P, Nicolai BM (2003) Predication of moisture loss across the cuticle of apple (*Malus sylvestris* supsp. *Mitis* (Wallr.)) during storage: part 2. Model simulations and practical applications. Postharvest Biol Technol 30:89-97.

Zheng WW, Zhai H, Yuehua H, Zhang J, WangX (2005) Analysis of the changes of calcium content of several apple cultivars in developing season. Scientia Agricultura Sinica 38(11):2296-2300.

Volume 53(1):27-32, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Study of intracultivar variation among main Iranian olive cultivars using SSR markers

Zahra Noormohammadi[1, 2]*, Mehdi Hosseini-Mazinani[2], Isabel Trujillo[3], Angjelina Belaj[4]

[1]Biology Department, School of Basic sciences, Science and Research Branch, Islamic Azad University (SRBIAU), Poonak, Tehran, Iran, [2]National Institute of Genetic Engineering & Biotechnology (NIGEB), Tehran, Iran, [3]Agronomy Department, Cordoba University, Cordoba, Spain, [4]C.I.F.A. "Alemeda del Obispo", IFAPA, Cordoba, Spain

ABSTRACT Three Iranian olive cultivars of Geloleh, Shengeh and Rowghani with commercial interest are distributed in 3 provinces in North of Iran. Fifty one accessions belonging to these 3 olive cultivars were screened by 13 microsatellite markers revealing high genetic variability both within and between cultivars. In total, 54 alleles were detected with a mean number of 4.2 alleles per locus. Six unique allelic patterns were observed. Heterozygosity ranged from 0.00 to 1.00 while the mean number of polymorphic information content (PIC) was 0.51. The existence of homonyms, synonyms or mislabeling as well as intracultivar polymorphism was showed by allele differences between olive accessions studied. The phenogram obtained by UPGMA clustering showed variability among as well as between cultivars.
Acta Biol Szeged 53(1):27-32 (2009)

KEY WORDS

Genetic variability
Iran
olive
SSRs

Olive (*Olea europaea* L.) is a subtropical species typical of the Mediterranean basin and it most likely originated from the Near-East during the Chalcolithic period (5700-5500 years B.P; Zohary and Hopf 1994).

Archaeological findings revealed that olive cultivation in Iran dates back to 2000 years ago (Sadeghi 1992). At present olive cultivars are cultivated mainly in the North of Iran, which is characterized by Mediterranean climatic condition. In the last ten years, olive plantation has grown in Iran and currently, 95000 hectares of olive orchards produce about 6500 tons of olive oil annually. Although a large number of olive accessions are growing in Iran, there have been few reports on morphological, cytogenetical and molecular characteristics of these accessions (Samaee et al. 2003; Hosseini-Mazinani et al. 2004; Noormohammadi et al. 2007; Omrani-Sabbaghi et al. 2007; Sheidai et al. 2007).

Discrimination of varieties based on morphology evaluation is limited by effect of environmental conditions, the need for extensive observations of mature plants and requirement of well-trained staff (Belaj et al. 2001). Therefore, more comprehensive studies using reliable markers are needed to gain a better understanding of the levels of genetic diversity in olive cultivars, which may be of use in the cultivars identification.

Different molecular techniques such as isozymes (Trujillo et al. 1995) Random Amplified Polymorphic DNA (RAPDs; Wiesman et al. 1998; Mekuria et al. 1999; Belaj et al. 2001; Besnard et al. 2001) Amplified Fragment Length Polymorphism (AFLP; Angiolillo et al. 1999) and Simple Sequence Repeat (SSR; Rallo et al. 2000; Sefc et al. 2000; Bandlej et al. 2002) have been increasingly used to characterize the olive cultivars.

Microsatellites are useful because they are abundant, uniformly distributed, highly polymorphic, codominant and amenable to automation (Morgante and Olivieri 1993; Powell et al. 1996; Rafalski et al. 1996). Microsatellite markers have been proven to be very suitable markers for fingerprinting and revealing the genetic diversity in olive cultivars (Cipriani et al. 2002; De la Rosa et al. 2002; Khadari et al. 2003; Belaj et al. 2004; Diaz et al. 2006).

The present study tries to characterize three important Iranian olive cultivars of Geloleh, Shengeh and Rowghani which cultivated in three provinces of Gilan, Zanjan and Ghazvin reporting the available genetic polymorphism both within and between cultivars.

Materials and Methods

Plant materials and DNA extraction

Fifty one accessions belonging to 3 Iranian olive cultivars (Geloleh, Shengeh and Rowghani) were used in the molecular study (Table 1). Naming of cultivars have been based on common morphological traits ('Geloleh' = round fruit) and practical utility (Rowghani = oily cultivar). These cultivars were identified on the basis of morphological characteristics (Sadeghi 1992). Trees were sampled from seven different locations randomly selected in Gilan, Zanjan and Ghazvin

Accepted June 2, 2009
*Corresponding author. E-mail: marjannm@yahoo.com, z-nouri@srbiau.ac.ir

Table 1. Cultivar accession included in the study with tree code, source of material, geographical diffusion and use of fruit, ordering number (Nr).

Nr	Cultivar accession	Tree**	Source of materials	Geographical diffusion	Use of fruits*
1	GELOLEH	*1136, 1127, 1147,* 1139	Harzevil	Gilan	O
2	GELOLEH	*501, 502, 1158, 389,* 391, 387, 393	Ettka Garden	Gilan	O
3	GELOLEH	*497*	BahramAbad	Ghazvin	O
4	GELOLEH	*1117, 1122,* 1118	Manjil	Gilan	O
5	GELOLEH	*316,* 313, 317	Motahari Garden	Zanjan	O
6	ROWGHANI	*1048, 1050,* 1049	Research Garden	Gilan	O&T
7	ROWGHANI	*209,* 208	BahramAbad	Ghazvin	O&T
8	ROWGHANI	*119, 112, 122,* 123	Vakhman	Ghazvin	O&T
9	ROWGHANI	*367, 376*	Ettka Garden	Gilan	O&T
10	ROWGHANI	332, 331, 321	Motahari Garden	Zanjan	O&T
11	SHENGEH	*363*	Ettka Garden	Gilan	O&T
12	SHENGEH	*1089, 1097, 1090,* 1085 *1086, 1096, 1098,*1119 *263, 259, 1094,* 1103 *1083,1102, 1104, 1082*	Research Garden	Gilan	O&T
13	SHENGEH	*1115,* 1116	Manjil	Gilan	O&T

* O (oil), T (Table olive) and O&T (Oil and Table olive)
** Italic shows that accession which studied by 13 microsattelite markers.

provinces in North of Iran. In order to reduce the number of replication of each genotype, a primary screening was performed on 51 olive accessions by using five high polymorphic SSR markers (ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA16, ssrOeUA-DCA18 and UDO99-043). Representative of those accessions which showed the same allelic profiles involved in further studies while other samples eliminated. Therefore the number of genotypes reduced into 32 for more studies.

Total genomic DNA was extracted from fresh leaves using the CTAB method (Murry and Tompson 1980) with modification described by De la Rosa and coworker (2002).

Microsatellite assay

Thirteen microsatellite markers of ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA11, ssrOeUA-DCA15, ssrOeUA-DCA16, ssrOeUA-DCA18 (Sefc et al. 2000) UDO99-011, UDO99-019, UDO99-043, UDO99-024 (Cipriani et al. 2002) and GAPU59, GAPU71B, GAPU101 (Carriero et al. 2002) were used for studying genetic polymorphism in 32 selected olive accessions. Amplification of microsatellites was performed in PCR reactions in a total volume 20µl, containing 2 ng genomic DNA, 1X supplied PCR buffer (Biotools, Spain) 200µM of each dNTP (Roche), 0.25 unit of Taq DNA polymerase (Biotools, Spain) and 0.2 µM of forward (fluorescently labeled) and reverse primers. The amplifications were carried out on a thermal cycler (Perkin-Elmer-9600) programmed with a denaturation at 94°C for 5 min, 35 cycles of 94°C for 20 s, the annealing temperature 50°C for 30 s and 72°C for 30 s and final extension at 72°C for 7 min. Finally, the analysis was carried out on an automatic capillary sequencer ABI 3130 Genetic Analyzer (Applied Biosystems/HITACHI) using fluorescent dyes, and fragment sizes were determined using internal standards.

Data analysis

The peaks present in genotypes were recorded for each of the thirteen microsatellite loci using Genotyper 3.7 computer software (Applied Biosystems). Observed heterozygosity (H_0) was obtained as the ratio of the heterozygous individuals to the total number of genotypes per locus, expected heterozygosity (H_E) (Nei 1987), Polymorphic information content (PIC) (Botstein et al. 1980) and null alleles frequency (r) (Brookfield 1996) were also calculated.

Genetic distances between all pairwise combinations of the accessions were calculated using different similarity measures including Dice and Jaccard's coefficients. Grouping of the genotypes was determined by using different clustering methods including UPGMA (unweighted paired group mean using average), Single linkage and WARD (minimum spherical cluster) methods as well as ordination based on principal coordinate analysis (PCO) (Ingrouille 1986; Chatfield and Collin 1995). Cophenetic correlation was determined for different clustering methods. NTSYS-pc version 2.02 (Rohlf 1998), Cervus version 2.0 (Marshall et al. 1998) softwares were used for statistical analyses.

Results

SSR diversity in three Iranian olive cultivars

The initial screening with five primers (ssrOeUA-DCA3, ssrOeUA-DCA9, ssrOeUA-DCA16, ssrOeUA-DCA18 and

Table 2. Allele size, number of alleles, unique alleles and heterozygosity indices for studied cultivars in 13 SSR primers.(H_o) observed heterozygosity,(H_E) expected heterozygosity, (PIC) Polymorphic Information Content.

Locus	Size range	No. alleles	No. unique alleles	No. unique allele patterns	H_o	H_E	PIC	Probability of null alleles
ssrOeUA-DCA3	229/253	6	1	2	0.93	0.80	0.75	-0.0917
ssrOeUA-DCA9	170/207	8	2	2	0.84	0.78	0.74	-0.0395
ssrOeUA-DCA16	122/178	4	0	1	0.61	0.60	0.52	-0.0134
ssrOeUA-DCA18	162/180	4	0	1	0.46	0.43	0.38	-0.0363
UDO99-043	170/216	6	2	5	0.68	0.65	0.60	-0.0456
ssrOeUA-DCA11	142/178	4	0	0	1.00	0.74	0.68	-0.1577
ssrOeUA-DCA15	243/263	3	0	0	0.10	0.46	0.41	+0.6274
UDO99-011	114/130	5	1	1	0.97	0.68	0.62	-.02031
UDO99-019	129	1	0	0	0.000	0.000	0.000	0.000
UDO99-024	166/189	3	0	0	0.56	0.55	0.46	-0.0109
GAPU59	206/216	2	0	0	0.40	0.33	0.27	-0.1117
GAPU71B	121/135	3	0	1	0.65	0.63	0.55	-0.0089
GAPU101	191/217	5	0	0	0.75	0.72	0.67	-0.0205
Total*	-	54	6	13	0.61	0.57	0.51	-

*Numbers of H_o, H_E, PIC are mean values.

UDO99-043) revealed the presence of the same allelic profiles among different trees (replications) of the same cultivars. So, among 51 accessions, 19 trees could be considered as duplications of the plant materials and they were excluded from further analysis. All used thirteen microsatellite markers (except UDO99-019) were polymorphic, revealing the presence of 54 alleles in all 3 cultivars analyzed. The number of alleles in each locus varied from one (UD99-O19) to eight (DCA9) with an average number of 4.2 alleles per locus (Table 2).

Under "Hardy Weinberg" equilibrium, the observed heterozygosity varied from 0.000 in monomorphic locus of UDO99-019 to 1.00 in DCA11 locus with an average value of 0.61. The observed Heterozygosity at all loci was higher than expected heterozygosity except DCA15 locus with high frequency of null alleles (Table 2). The study of allelic polymorphism obtained in the present work allows discrimination of 82% of the olive accessions analyzed by producing unique genotype profiles (Table 2). The UDO43 and DCA9 loci revealed the highest number of unique alleles (2) while some loci did not show any unique alleles (for example DCA16, DCA18 and GAPU59; Table 2). Calculated PIC values ranged from 0.000 to 0.75 in 13 loci with average of 0.51. DCA3 locus showed highest PIC value while DCA19 showed lowest PIC value.

Genetic relationships

Different similarity coefficients determined among the cultivars studied, showed the highest value of similarity (r = 1.0) between three pairs of accessions such as Shengeh-263 and Shengeh-363 and also Rowghani-1050 and Rowghani-209.

Similarity matrices obtained from cultivars studied were employed for elucidating the genetic relationships among olive cultivars by different clustering methods, including UPGMA, single linkage and WARD (Fig 1). The cophenetic coefficients determined for different clustering methods revealed the highest value for UPGMA (r = 0.81) indicating a good fit of the original data to the clustering dendrogram.

Discussion

The high level of variation in average number of alleles per locus (4.2) was observed in olive cultivars. The same result has also been reported by other workers (Rallo et al. 2000; Carriero et al. 2002; De la Rosa et al. 2002; Khadari et al. 2003; Belaj et al. 2004). They have reported average values of 7.5, 6.4, 5.7, 7.4 and 5.2 alleles per locus related to different number of loci in their studies. Variation reported in the number of alleles in olive cultivars by different workers may be related to variation in the loci studied as well as the number of genotypes and their localities (Lopes et al. 2004).

The heterozygosity deficiency has been found in DCA15 locus to be non-significant based on χ^2 test ($p < 0.001$) might be due to the presence of null alleles. A possible explanation of the deficit in amount of observed heterozygosity may be the occurrence of null alleles at this locus (Ishibashi et al. 1996). The presence of null alleles is a consequence of sequence polymorphisms in the flanking regions of the locus due to point mutations or insertion/deletions (Jones et al. 2003). Higher expected values of heterozygosity were also reported by Lopes et al. (2004), which were related to the occurrence of the null alleles.

The study of allelic polymorphism discriminated 82% of the olive accessions analyzed by producing unique genotype profiles. Therefore, the olive cultivars studied can be well discriminated by using the above said indices due to the

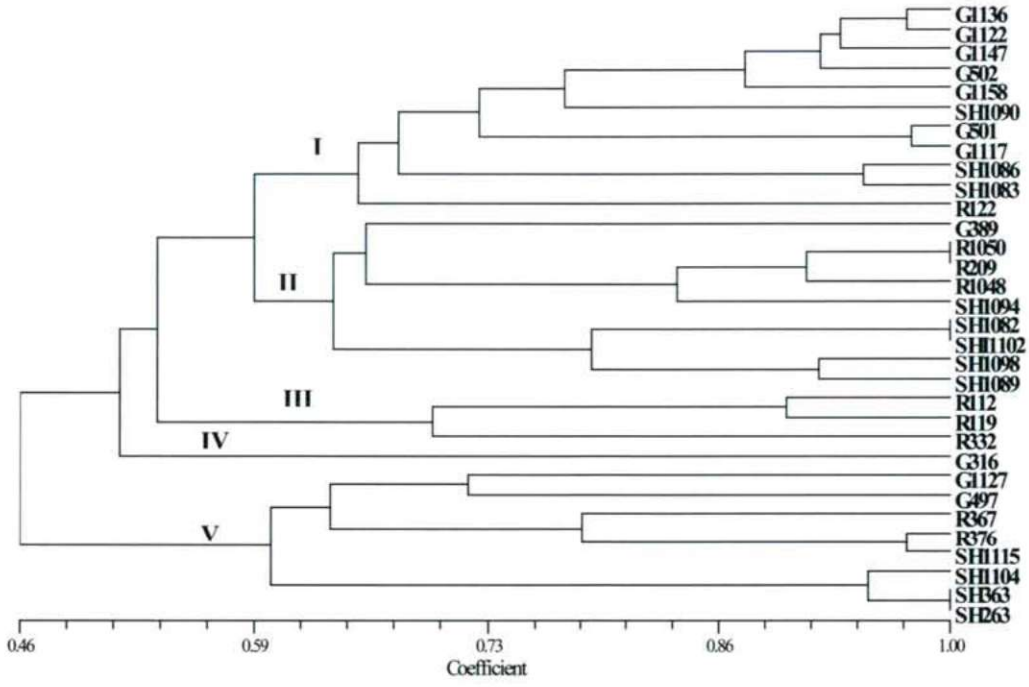


Figure 1. UPGMA dendrogram of Iranian olive cultivars based on Dice's coefficient. Abbreviations: G (GELOLEH), SH (SHENGEH), R (ROWGHANI).

presence of a high amount of genetic variability among these three cultivars.

Calculated PIC values with average of 0.51 are close to the values observed by Bandelj et al. (2004). It has been suggested that PIC values > 0.5 are informative markers while loci with PIC values > 0.7 are suitable for genetic mapping (Bandelj et al. 2004). Therefore in the present study eight loci may be considered informative while four loci (DCA3, DCA9, UDO11 and GAPU101) may be used in genetic mapping of the cultivars studied (Table 2).

By using UPGMA method, five major groups/clusters were identified in cluster analysis. The first major cluster is mainly comprised of Geloleh accessions, three accessions of 'Shengeh' and one accession of 'Rowghani'. The second major cluster consists of two subclusters. Accessions of Geloleh-389, Rowghani-1050, 209, 1048 and 'Shengeh-1094' formed the first subcluster while second subcluster consists of 4 'Shengeh' accessions (Fig. 1). The third major cluster is comprised of accessions of 'Rowghani-112', 'Rowghani-119' and 'Rowghani-332', while one accession of 'Geloleh-316' formed the forth cluster. The fifth major cluster contains two subclusters, some accessions of 'Geloleh', 'Shengeh' and 'Rowghaňi' form the first subcluster while 3 accessions of 'Shengeh' are placed in the second subcluster. Two accessions 'Rowghani- 376' and 'Shengeh-1115' differed only in one locus (data not shown). These accessions with similar genotypes and different denomination are suggested to be synonymous or mislabeled.

In the present work, all five clusters included accessions are from three Northern provinces of Iran (Gilan, Zanjan and Ghazvin) without geographical separation. This may show material exchanges occurred between these provinces by local gardeners. Meanwhile, it is suggested that olive cultivars might be renamed or misnamed in new locality which were cultivated.

The stability of the groups was also confirmed by partitioning the variants of data sets using PCO. Generally, PCO plot supported the clustering results obtained (Fig. 2).

Generally accessions of 3 olive cultivars studied are distributed in different clusters possibility due to their genetic variability or misnaming. These cultivars are very common in the North of Iran and their denominations are complicated because of morphological similarity. Homonymy is one of the problems in Iranian olive germplasm as much as occur in Mediterranean's cultivars. The main reason may be come from denominating cultivars based on common morphological traits, particularly of fruit or practical use of cultivar like Geloleh which means round fruit and Rowghani because of producing olive oil. Therefore generic names of Iranian olive cultivars specially these 3 main olive cultivar include different genotypes and may be considered homonyms or mislabeling. Discrimination of homonymous cases in olive germplasm has also been reported by using SSRs and other molecular markers by other workers (Belaj et al. 2001; Khadari et al. 2003). Intracultivar variations have also been reported in 'Shengeh' by using morphological characters (Hosseini-Mazinani et al.

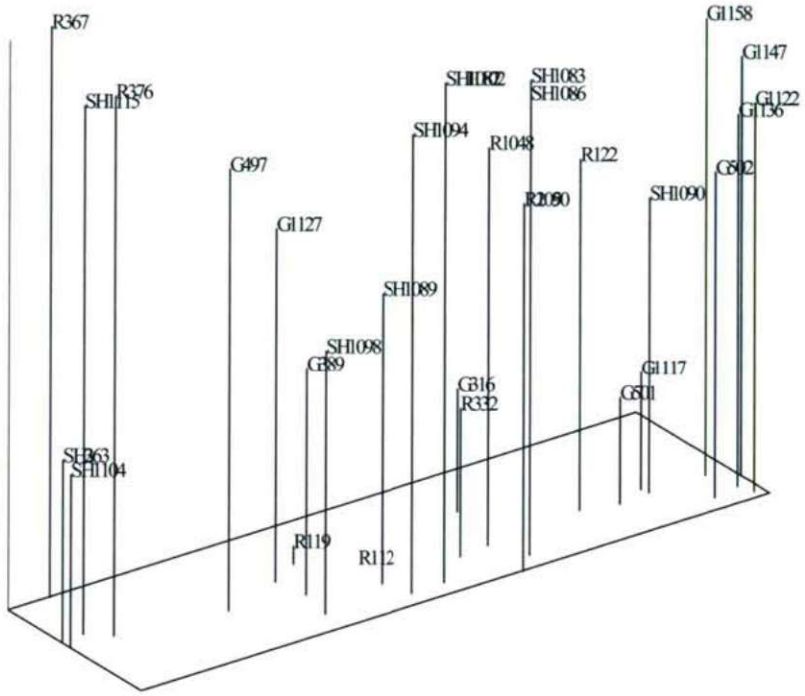


Figure 2. PCO ordination of the studied olive cultivars based on SSR markers.

2004). Omrani-Sabbaghi et al. (2007) also has been reported the existence of intracultivar variation in some Iranian olive cultivars.

In conclusion, this study shows that SSR markers are a powerful tool for cultivar identification and analysis of genetic structure. High information content of the markers enables characterization and discrimination of olive cultivars only with combination a few loci. The most of microsatellite markers used in this study were very informative in the 3 Iranian olive cultivars analyzed. Genetic characterization of a larger number of genotypes will help in identifying potentially synonymous and homonymous cultivars, which will be very useful in germplasm management.

Acknowledgments

This research was supported by NIGEB Grant Number 241.

References

Angiolillo A, Mencuccini M, Baldoni L (1999) Olive (*Olea europaea* L.) genetic diversity assessed by Amplified Fragment Length Polymorphisms. Theor Appl Genet 98:411-421.

Bandelj D, Jakse J, Javornik B (2004) Assessment of genetic variability of olive variaties by microsatellite and AFLP markers. Euphytica 136:93-102.

Bandelj D, Jakse J, Javornik B (2002) DNA fingerprinting of olive varieties by microsatellite markers. Food Technol Biotechnol 40(3):185-190.

Belaj A, Cipriani G, Testolin R, Rallo L, Trujillo I (2004) Characterization and identification of the main Spanish and Italian olive cultivars by simple-sequence-repeat markers. HortScience 39(7):1557-1561.

Belaj A, Trujillo I, De la Rosa R, Rallo L, Gimenez M J (2001) Polymorphism and discriminating capacity of randomly amplified polymorphic markers in an olive germplasm bank. J Amer Soc Hort Sci 126:64-71.

Besnard G, Breton C, Baradat P, Khadari B, Berville A (2001). Cultivar identification in olive (*Olea europaea* L.) based on RAPDs. J Am Soc Hort Sci 126:668-675.

Botstein D, White R L, Skolnick M, Davis RW (1980) Construction of genetic linkage map in man using restriction fragment length polymorphism. Am J Hum Genet 32:314-331.

Brookfield J F Y (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. Mol Ecol 5:453-455.

Carriero F, Fontanazza G, Cellini F, Glorio G (2002) Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). Theor Appl Genet 104:301-307.

Chatfield C and Collins A J (1995) Introduction to Multivariate Analysis. Chapman and Hall, London.

Cipriani G, Marruzzo M T, Marconi R, Cimato A, Testolin R (2002) Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal Polymorphism within ancient cultivars. Theor Appl Genet 104(2-3):223-228.

De la Rosa R, James C, Tobutt K R (2002) Isolation and characterization of polymorphic microsatellite in olive (*Olea europaea* L.) and their transferability to other genera in the *Oleaceae*. Primer note. Mol Ecol Notes 2:265-267.

Diaz A, De la Rosa R, Martin A, Rallo P (2006) Development, characterization and inheritance of new microsatellites in olive (*Olea europaea* L.) and evaluation of their usefulness in cultivar identification and genetic relationship studies. Tree Genetics & Genomes 2:165-175.

Hosseini-Mazinani S M, Samaee S M, Sadeghi H, Caballero JM (2004) Evaluation of olive germplasm in Iran on the basis of morphological traits: Assessment of Zard and Rowghani cultivars. Acta Hort 634: 145-151.

Ingrouille M J (1986) The construction of cluster webs in numerical taxonomic investigations. Taxon 35:541-545.

Ishibashi Y, Saitoh T, Abe S, Yoshida C (1996) Null microsatellite alleles due to nucleotide sequence variation in the grey-sided vole *Clethrionomys rufocanus*. Mol Ecol 5:589-590.

Jones AG, Ardren W R (2003) Methods of parentage analysis in natural populations. Mol Ecol 12:2511-2523.

Khadari B, Breton C, Moutier N, Roger J P, Besnard G, Berville A, Dosba F (2003) The use of molecular markers for germplasm management in a French olive collection. Theor Appli Genet 106:521-529.

Lopes MS, Mendonca D, Sefc K M, Gil F S, Da Camara Machado A (2004) Genetic evidence of intra-cultivar variability within Iberian olive cultivars. Hortscience 39(7):1562-1565.

Marshall TC, Slate J, Kruuk L, Pemberton J M (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639-655.

Mekuria GT, Collin G C, Sedgley M (1999) Genetic variability between different accessions of some common commercial olive cultivars. J Hort Sci Biotechnol 74(3):309-314.

Morgante M, Olivieri A M (1993) PCR-amplified microsatellites as markers in plant genetics. The Plant Journal 3:175-182.

Murry M G, Tompson W F (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321-4325.

Nei M (1987) Genetic distance and molecular phylogeny. In Ryman N and F Utter, eds., Population genetics and fishery management. pp. 193-223, University of Washington Press, Seattle.

Noormohammadi Z, Hosseini-Mazinani M, Belaj A, Trujillo I, Rallo L, Sadeghizade M (2007) Identification and Classification of Main Iranian Olive Cultivars Using Microsatellite Markers. Hortscience 42(7):1545-1550.

Omrani-Sabbaghi A, Shahriari M, Falahati-Anbaran M, Mohammadi SA, Nankali A, Mardi M, Ghareyazie B (2007) Microsatellite markers based assessment of genetic diversity in Iranian olive (*Olea europaea* L.) collections. Sci Hortic 112(4):439-447.

Powell W, Morgante M, Andre C, Hanafey M, Tingey J, Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding 2:225-238.

Rafalski J A, Vogel JM, Morgante M, Powell W, Andre C, Tingey SV (1996) Generating and using DNA markers in plants. In Birren B, E Lai, eds., No mammalian genomic analysis. A practical guide. Academic Press, San Diego, pp. 75-134.

Rallo P, Dorado G, Martin A (2000) Development of Simple Sequence Repeats (SSRs) in olive tree (*Olea europaea* L.). Theor Appl Genet 101:984-989.

Rohlf F J (1998) NTSYS-pc. Numerical taxonomy and multivariate analysis system. Version 2.00. Exeter Software, Setauket, N.Y.

Sadeghi H (1992) Cultivation, preservation and harvesting of olive. Eds., The publication of Ministry of Agriculture, Tehran, Iran.

Samaee S M, Shobbar Z S, Ashrafi H, Hosseini-Mazinani M, Sheidai M (2003) Molecular characterization of olive germplasm in Iran by use of Random Amplified Polymorphic DNA (RAPD): Correlation with phenotypic studies. Acta Hort 623:169-175.

Sefc KM, Lopes M S, Mendoca D, Rodrigues Dos Santos M, Laimer Da Camara Machado M, Da Camara Machado A (2000) Identification of microsatellite loci in olive (*Olea europaea* L.) and their characterization in Italian and Iberian olive trees. Mol Ecol 9:1171-1173.

Sheidai M, H- Shahreiyari Z, Noormohammadi Z, Parsian H, Farahanei F (2007) Study of Genetic diversity in Some Olive (*Olea europaea* L.) Cultivars by Using RAPD markers. Pakistan Journal of Biological Science 10(17):2972-2975.

Trujillo I, Rallo L, Arus P (1995) Identifying olive cultivars by isozyme analysis. J Amer Soc Hort Sci 120:318-324.

Wiesman Z, Avidan N, Lavee S, Quebedeaux B (1998) Molecular characterization of common olive varieties in Israel and the West Bank using randomly amplified polymorphic DNA (RAPD) markers. J Am Soc Hort Sci 123:837-841.

Zohary D, Hopf M (1994). Domestication of plants in the Old World. Second edition. Oxford Clarendon Press, pp. 137-442.

Volume 53(1):33-37, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

The value of honey bees (*Apis mellifera*, L.) as pollinators of summer seed watermelon (*Citrullus lanatus colothynthoides* L.) in Egypt

El-Kazafy A. Taha[1], Yousry A. Bayoumi[2]*

[1]Economic Entomology Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, Egypt, [2]Horticulture Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, Egypt

ABSTRACT The value of honey bee, *Apis mellifera* L., as pollinator of summer seed watermelon plants, *Citrullus lanatus colothynthoides* L. was studied. The highest percentage of opened flowers, number of bees/m²/min and amount of trapped pollen/ colony/h were recorded between 9.00 and 10.00 h, with significant (P<0.01) correlations between them. Eleven insect species belonging to eleven families and five orders were recorded as pollinators on summer seed watermelon crop, and A. mellifera L., was the predominant species. One hectare of summer seed watermelon could produce 10.47 kg of honey per season. Open pollination treatment produced the highest number of mature fruits and seed yield as compared with caged plants without any insect visitors which did not produce any fruits at all. It could be recommended to move the honey bee colonies to summer seed watermelon plantations during its flowering period to build-up the colonies and increase seed yield.
Acta Biol Szeged 53(1):33-37 (2009)

KEY WORDS

Honey bees
Summer seed watermelon
Citrullus lanatus colothynthoides
pollen
pollination

Pollination plays an important role in flowering plant reproduction and fruit set for wild plant communities (Corbet et al. 1991; Buchmann and Nabhan 1996). Estimates showed that up to 90% of all flowering plant species rely on pollination by insects such as bees (Richards 1986; Buchmann and Nabhan 1996). Agricultural crops often depend, at least in part, on unmanaged pollinator for their productivity (Klein et al. 2003; Kremen et al. 2002, 2004; Ricketts 2004; Ricketts et al. 2004). Watermelon is obligately dependent on multiple bee visits for pollination (Stanghellini et al. 1997). Although insect species such as beetles and solitary bees (Jaycox et al. 1975) and flies and butterflies (Shawer et al. 1981) have been recorded as pollinators of cucurbits, it is generally recognized that honey bee (*A. mellifera* L.) is the most important pollinators in commercial crop production (Free 1993; Delaplane and Mayer 2000).

Animal pollinators are thought to contribute between 15% and 30% of global food production (Roubik 1995). The annual value added to US crops production by honey bees A. mellifera is estimated to be $9.3 billion (Robinson et al. 1989) and $5-14 billion (Southwick and Southwick 1992). In Europe, pollination by honey bees is worth approximately €4.25 billion, and pollination by other taxa worth approximately €0.75 billion (Borneck and Merle 1989).

The dependency of fruit set on insect pollination is well studied in cucumber (*Cucumis sativa* L.) and watermelon (*Citrullus lanatus* (Thunb) Matsun & Nakai) (Free 1993), and squash (*Cucurbita pepo* L.; Shawer et al. 1981). Open pollination and honey bees provided during the whole flowering period treatments produced the highest number of mature fruit and seed yield, while squash caged without bees and other insect visitors did not produce any fruits (Shawer et al. 1981). Stanghellini et al. (1997) noted that there was 100 percent abortion for flowers receiving no entomophilous visitation, and significant abortion rates by flowers receiving low bee visit numbers emphasizing the need for active transfer of pollen in these crops by insect pollinators.

Summer seed watermelon (*Citrullus lanatus colothynthoides* L.) plants have been grown in considerable areas in scattered locations throughout Egypt; 34386 hectares in Lower Egypt, 185 hectares in Middle Egypt, 517 hectares in Upper Egypt and 4179 hectares in El-Noubaria, El-Beheira Governorate (Anonymous 2005). It is obligately rely on cross-pollination by insects, especially honey bees. In the newly reclaimed lands, the insect pollinators are too little; therefore, crop production is not economic. The present investigation aimed to throw the light on the foraging behavior of honey bees and their effects as pollinators on summer seed watermelon flowers.

Materials and Methods

This investigation was carried out on summer seed watermelon farm (11 hectares) in Dessouk district, Kafr El-Sheikh Governorate, Egypt from mid June till end of July 2006 (the

Accepted May 25, 2009
*Corresponding author. E-mail: ybayoumi2002@yahoo.com.sq

Table 1. Phenology of summer seed watermelon flowering.

Items	Total number of flowers		Sex ratio	Blooming period		Pollen load	
	m^2	Hectare		Starting	Ending	Weight (mg)	Color
	446.59	4464113.64	1 :7.5	10/6	31/7	5.59	Yellowish orange

Table 2. A list of insect pollinators on summer seed watermelon plants at Dessouk region, Kafr El-Sheikh Governorate during 2006 season.

Order	Family	Scientific name	Percentage
Hymenoptera	Apidae	*Apis mellifera* L.	61.52±0.42
	Andrenidae	*Andrena ovatula* K.	5.77±0.33
	Vespidae	*Polistes foederata* Kohl.	1.93±0.06
Diptera	Muscidae	*Musca domestica* L.	5.77±0.08
	Syrphidae	*Syrphus corollae* F.	5.77±0.16
	Tabanidae	*Tabanus taeniola* Pol.	7.68±0.16
Odonata	Aeschnidae	*Hemianax ephippiger*	3.85±0.1c2
	Agrionidae	*Ischnura senegalensis* Ramb.	1.93±0.24
Lepidoptera	Hesperidae	*Gegenes nastrodamus* F.	1.92±0.33
	Lycaenidae	*Tarucus* sp.	1.93±.041
Coleoptera	Coccinellidae	*Coccinella undecimpunctata* L.	1.93±0.16

blooming period of the crop). Two apiaries (250 colonies) surrounded the experimental farm at distances between 150 and 300 m. Sixteen nucleus colonies (each had about 10000 bees) of hybrid Carniolan honey bees were moved to the experimental farm to study the following aspects:-

Foraging behavior of honey bees

To study the behavior of honey bees for pollen collection, three colonies were provided with pollen traps at the peak of emblossoming for two weeks. The trapped pollen loads were collected hourly, from 07.00 to 15.00 h then weighted. The number of opened flowers/m^2, sex ratio and number of bees/m^2/min were counted at the same times. Mean weight of one pollen load was determined.

Survey of insect pollinators

At the peak of flowering, all insect species visited the plants and their flowers were collected by taking 20 double sweeps with sweep net randomly in the field. The collections were transferred to the laboratory for counting and identification.

Nectar secretion and sugar concentration

Certain flowers were bagged in the early morning before anthesis to prevent bees from nectar collecting. The nectar was collected by capillary tube from bottom of the flowers. Sugar concentration was estimated by pocket refractometer immediately in the field. The amount of expected honey per hectare was gravimetrically calculated from the following equations:

$$\text{Amount of nectar/hectare} = \text{Amount of nectar/flower} \times \text{No. flowers/hectare}$$

$$\text{Amount of sugar/hectare} = \text{Amount of nectar/hectare} \times \text{Nectar sugar concentration}$$

$$\text{Amount of expected honey/hectare} = \frac{\text{Amount of sugar/hectare}}{\text{Sugar concentration in honey (80\%)}}$$

Efficiency of honey bee as pollinator

To determine the effectiveness of insect pollinators on seed yield, two treatments were performed. In the first treatment, plants were left to open pollination, from which three-square meters were randomly selected to procedure the measurements. In the second one, other three square meters were isolated from insect pollinators by using wooden cages (1 ×l ×1 m) covered with wire screen, which distributed on plants one week before starting the anthesis. The number of pistillate flowers and number of fruits/ m^2 was counted every two days. Successful fruiting index was evaluated according to Shawer et al. (1981), using the following equation:

$$\text{Successful fruiting index} = \frac{\text{Total number of fruits/plant}}{\text{Total number of pistillate flowers/plant}}$$

The number of mature fruits/m^2, percentage of aborted fruits, total weight of fruits/m^2 and mean fruit weight were recorded. Seeds were collected, dried in sun rays and weighed. The weight of 100 seeds (seed index) was estimated. The value added to the crop production by honey bees *A. mellifera* was calculated.

Statistical analysis

Data obtained were statistically analyzed according to Steel & Torrie (1980). Treatment means were compared by Duncan 's

Figure 1. Number of bees/m2/min and trapped pollen (g)/colony/h in relation to the time of day (h)

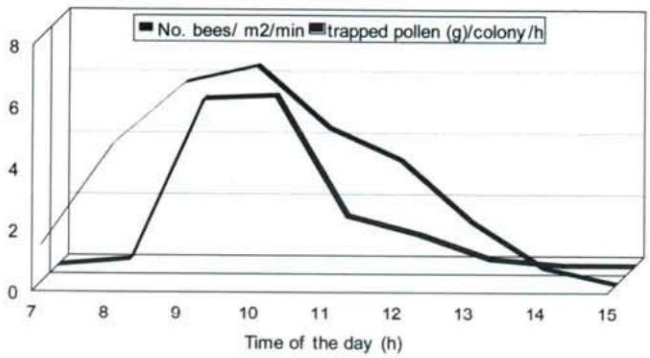
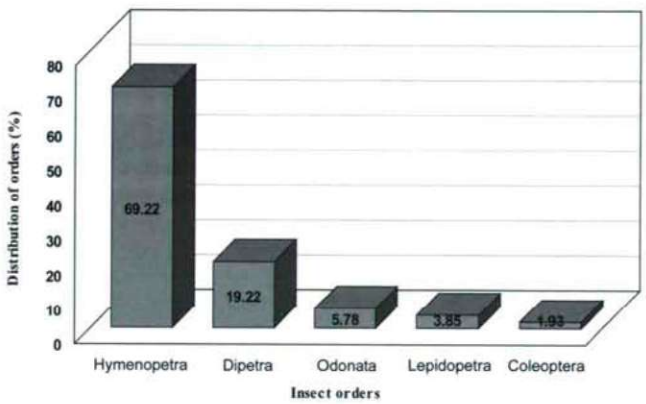


Figure 2. Distribution percentage of insect orders found on summer seed watermelon crop.



Multiple Range Test (Duncan 1955). Simple correlation was made by using "SPSS 10.0 for windows".

Results

Phenology of flowering

Summer seed watermelon plants start to grow in May, the blooming begins in the 2nd week of June and continues until the end of July. Blooming reached its maximum during the first half of July. The total numbers of flowers were 446.59 flowers/ m^2 and 4464113.64 flowers/hectare at the whole period of flowering. Male flowers greatly outnumber the female ones with sex ratio of 7.5 : 1 (male : female). The color of pollen loads is yellowish orange and the weight of one load averaged 5.59 mg (Table 1). The pollen grain was illustrated in Photo 1.

Foraging behavior of honey bee

The highest percentage (100%) of opened flowers, number of bees/m^2/min (6.55 & 7.08 bees) and amount of trapped pollen/colony/h (5.41 & 5.51g) were recorded during 9.00 and 10.00 h, respectively (Fig. 1). After 14.00 h, all flowers became closed and bee activity on flowers stopped.

Survey of insect pollinators

Data listed in Table 2 and illustrated graphically in Figure 2 showed that there were eleven insect species belonging to eleven families and five orders recorded as pollinators on the plants.

Nectar secretion and sugar concentration

As shown in Table 3, the amount of secretion nectar (mg/ flower) was higher in pistillate flower (14.49 mg) than in staminate one (9.52 mg).

Efficiency of pollination on fruit set

Data in Table 4 clearly show the effect of pollination on the seed yield of summer seed watermelon crop. The number of fruits/m^2 was 30.07 &0.00 fruits/m^2 and the successful fruiting index was 57.23 & 0.00 for open pollination and without pollination treatments, respectively. The mean numbers of mature fruits/m^2 were 8.56 &0.00 fruits, mean weight of mature fruit were 1386 & 0.00 g/fruit, seed yield/m^2 were 243.79 & 0.00 g/m^2 and the seed index was 15.33 g/100 seed for open pollination and without pollination treatments, respectively.

Discussion

The foraging of honey bee on summer seed watermelon plants depend mainly on percentage of opened flowers that related to the time of day. Similar results were recorded on squash by Shawer et al. (1981) who found that the activity of bees to visit flowers

Table 3. Amount of secreted nectar and its sugar concentration in summer seed watermelon flowers.

Expected honey / hectare (kg)	Sugar/hectare (kg)	Nectar/ hectare (kg)	Sugar concentration (%)	weight of nectar (mg) / flower	No. flowers Hectare	m^2	Flower sex
8.64	6.90	37.49	18.45	9.52	3938923.80	394.05	Staminate
1.83	1.48	7.69	19.24	14.64	525189.84	52.54	Pistillate
10.47	8.36	45.18	-	-	4464113.64	446.59	Total

Table 4. Effect of pollination on number of female flowers and fruits/m², number of mature of fruits/m², percentage of fruit aborted, average weight of mature fruit and seed yield of seed watermelon crop.

Treatments	No. female flowers/m²	No. fruits / m²	Successful fruiting index	No. mature fruits/m²	Mature fruit weight (g)	Seed yield (g/m²)	Seed index (g/100 seed)
Open pollination	52.54	30.07[a]	57.23[a]	8.56[a]	1386[a]	243.79[a]	15.33[a]
Without pollination	53.54	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Significant	N.S	**	**	**	**	**	**

_ N.S and ** indicate no significant and P<0.01, respectively.

Table 5. Correlation coefficient values for opened flowers (%), number of bees/m²/min and trapped pollen (g)/colony/h.

Comparisons	r – values
No. bees/m²/min x Opened flowers %	0.74*
Trapped pollen (g)/colony/h x Opened flowers %	0.95**
Trapped pollen (g)/colony/h x No. bees/m²/min	0.86**

- * and ** indicate P<0.05 and P<0.01, respectively.

reached its peak near 9.00 h (2.0 & 3.0 visits/ min) for staminate and pistillate flowers, respectively, after which the number of visits decreased and completely disappeared at 12.00 h at the same period of closing the flowers. Highly significant correlations were found between opened flowers percentage and each of number of bees/m²/min and trapped pollen/colony/h, and between trapped pollen/colony/h and number of bees/m²/min (Table 5). In this concern, DeGrandi-Hoffmann et al. (1991) observed that the greatest number of flowers/m² had also the most nectar and pollen per meter and attracted the greatest number of honey bees.

The dominant species visited the plants and their flowers were A. mellifera L. (61.52%), followed by male flies of Tabanus taeniola Pol. (7.68%). However, the insect orders were arranged according to their abundance on the crop as follows: Hymenoptera (69.22%), Diptera (19.22%), Odonata (5.78 %), Lepidoptera (3.85%) and Coleoptera (1.93%; Fig. 3). The obtained results are in harmony with those found by Shenishen and Shawer (1986) who concluded that, the distribution percentages of Hymenoptera were 67.59, 85.00, 100.00, 8.07, 24.32 and 74.79% on cabbage, cauliflower, lettuce, carrot, onion and leek crops, respectively. The previous results showed that, although the plurality of pollinators from different orders, the honey bees alone still formed more than sum of the other orders, which due to the high number of bees visited the flowers to collect pollen and/or nectar formed by plants. Bees worldwide stand out as the dominant pollinating group in nearly all geographic regions (Kearns 1992). However, Wilde et al. (1995) reported that in Poland during 1989-91, honey bee formed 66-75% of the population of pollinating insects on field beans (*Vicia faba* L.). In Australia, Rymer et al. (2005) noted that A. mellifera was the most common floral visitor to *Persoonia* flowers, being observed 3 to 10 times more frequently than native bees. On the other hand, flies (Diptera) were the second prevalent species as they represented 19.22% of all pollinators. These results are confirmed by the findings of Kevan and Baker (1983) who reported that, the more notable pollinating flies were belonging to the families: Bombyliidae, Syrphidae, Anthomiidae, Tachinidae, Calliphoridae and Musacidae.

Photo 1. Pollen grain of summer seed watermelon.

The total amount of nectar (kg/hectare) was higher in staminate flowers (37.49 kg) than in pistillate ones (7.69 kg). This may be due to the highest number of staminate flowers (393893.90 flowers/hectare) in comparison with pistillate ones (525189.84 flowers/hectare).

In respect of sugar concentration, it was higher in pistillate flowers (19.24%) than in staminate ones (18.45%). These results are in agreement with those obtained by Collison and Martin (1979) who found that pistillate cucumber flower produced more nectar and yielded slightly more sugar. The same results were recorded on squash (Shawer et al. 1981). From these results, it could be concluded that one hectare of summer seed watermelon could produce 10.47 kg of honey per season, which is sufficient for building-up one honey bee colony during the dearth period between clover flow and early cotton flow.

Aforementioned results proved the great importance of insect pollinators especially honey bee on fruit set in summer seed watermelon since caged plants without any entomophilous visitation did not produce any fruits at all. These results were confirmed by previous results on cucumber and watermelon (Stanghellini et al. 1997), cantaloup (Iselin et

al. 1974) and squash (Shawer et al. 1981; Couto et al. 1990; Skinner and Lovett 1992).

Gravimetrically, it could be theoretically calculate the value added to the crop production by honey bee *A. mellifera*. Non pollinated plants did not produce yield, while open pollinated produced 2436.93 kg seed per one hectare. The price of one kg seed equals 8.00 L.E., so crop of one hectare coasted 19495.44 L.E. As honey bee contributed by 61.52 % of all pollinators, then,

Value added by honey bee = 19495.44 ×61.52/100 =11993.59 L.E.

In this concern, Southwick and Southwick (1989) concluded that the annual value added to US crops production by *A. mellifera*, is estimated to be over 51 billion dollars.

Conclusion

Lastly, it could be concluded that, movement of honey bee colonies to summer seed watermelon plantations during its flowering period is recommended for economizing the cost of feeding, building-up the colonies and increase seed yield.

References

Anonymous (2005) Annual Report of Ministry of Agriculture, Egypt.

Borneck R, Merle B (1989) Essaie d'une evaluation de l'incidence économique de l'abeille pollinisatrice dans l'agriculture européenne. Apicata 24:33-38. (Abst.)

Buchmann SL, Nabhan GP (1996) The Forgotten Pollinators. Island Press, Washington DC.

Collison CH, Martin EC (1979) Behavior of honey bees foraging on male and female flowers of *Cucumis sativa*. J Apic Res 18:184-190.

Corbet SA, Williams IH, Osborne J (1991) Bees and the pollination of crops and wild flowers in the European community. Bee World 72:47-59.

Couto RH, Pereira JM, Couto LA (1990) Effects of pollination in *Cucurbita pepo* (summer squash). Cientifica 18(1):21-27. (c.f. Apic. Abst., 1498/92)

degrandi-Hoffmann G, Loper G, Thorp R, Eisikowitch D (1991) The influence of nectar and pollen availability and blossom density on the attractiveness of almond cultivars to honey bees. Acta Hortic 288:299-302.

Delaplane KS, Mayer DF (2000) Crop Pollination by Bees. CABI Publishing, UK.

Duncan BD (1955) Multiple Range and Multiple F. Test. Biometrics 11: 1-42.

Free JB (1993) Insect pollination of crops. 2nd Ed. Acad. Press, Londan, U.K., 544pp.

Iselin WA, Jensen MH, Spangler HG (1974) The pollination of melons in air inflated greenhouses by honey bees. Environ Entomol 3(4):664-666.

Jaycox ER, Guynn G, Rhodes AM, Vandemark JS (1975) Observation on pumpkin pollination in Illinois. American Bee Journal 115(4):139-140.

Kearns CA (1992) Anthophilous fly distribution across an elevation gradient. American Midland Naturalist 127:172-182.

Kevan PG, Baker HG (1983) Insects as flower visitors and pollinators. Annals Review of Entomology 28:407-445.

Klein A, Steffan-Dewenter I, Tscharntke T (2003) Pollination of *Coffea canephora* in relation to local and regional agroforestry management. J Appl Ecol 40:837-845.

Kremen C, Williams NM, Thorp RW (2002) Crop pollination from native bees at risk from agricultural intensifications. Proc Natl Acad Sci USA 99:16812-16816.

Kremen C, Williams NM, Bugg RL, Fay JP, Thorp RW (2004) Estimating the area requirements of an ecosystem service, crop pollination. Ecological Letters 7:1109-1119.

Richards AJ (1986) Plant breeding systems. Chapman and Hall, New York, USA.

Ricketts TH (2004) Do tropical forest fragments enhance pollinator activity in nearby coffee crops? Cons Biol 18:1-10.

Ricketts TH, Daily GC, Ehrlich PR, Michener CD (2004) Economic value of tropical forest to coffee production. Proc Natl Acad Sci U S A 101:12579-12582.

Robinson WS, Nowogrodzki R, Morse RA (1989) The value of honey bees as pollinators of U.S. crops. Part II. American Bee Journal 129(8): 477-487.

Roubik DW (1995) Pollination of cultivated plants in the tropics. FAO, Rome.

Rymer PD, Whelan HJ, Ayre DJ, Weston PH, Russell KG (2005) Reproductive success and pollinator effectiveness differ in common and rare *Persoonia* species (Proteaceae). Biol Cons 123:521-532.

Shawer MB, El-zawily AI, Metwally SM, Ghazy MM (1981) The efficiency of honey bees as pollinators of summer squash (*Cucurbita pepo* L.). Journal of Agriculture Research Tanta University 7(2):225-238.

Shenishen Z, Shawer MB (1986) Insect pollinators and seed set of certain vegetable crops. Proceedings of the 1st Horticulture Sciences Conference Tanta University, September 1:277-291.

Skinner JA, lovett G (1992) Is one visit enough? Squash pollination in Tennessee. American Bee Journal 132(12):815.

Southwick EE, Southwick LJR (1992) Estimating the economic value of honey bees (Hymenoptera, Apidae) as agricultural pollinators in the United States. J Econ Entomol 85(3):621-633.

Southwick LJR, Southwick EE (1989) A comment on value of honey bees as pollinators of U.S. crops. American Bee Journal 129(12):805-807.

Stanghellini MS, Ambrose JT, Schultheis JR (1997) The effects of honey bee and bumble bee pollination on fruit set and abortion of cucumber and watermelon. American Bee Journal 137(5):386-391.

Steel R G, Torrie JH (1980) Principles and procedures of statistics. 2 nd ed. pp. 120. Mc Graw-Hill, New York, USA.

Wilde J, Krukowski R, Smoczynski S, Bobrzecki J (1995) Effects of chemical protection of field bean on mortality of honey bees and amount of pesticides in honey and pollen. Pszczelnicze Zeszyty Naukowe 39(2): 97-107. (Abst.)

Volume 53(1):39-43, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

New chromosome number and unreduced pollen formation in *Achillea* species (Asteraceae)

Sheidai, M[1]*, Azanei, N.[1], Attar, F[2]

[1]Shahid Beheshti University, GC., Faculty of Biological Sciences, Tehran, Iran, [2]School of Biological Sciences, University of Tehran, Tehran, Iran

ABSTRACT Cytological studies were performed in 14 populations of 8 *Achillea* species growing in Iran. *A. eriophora*, *A. tenuifolia*, *A. oxyodonta*, *A. talagonica* and *A. biebersteinii* showed 2n = 2x = 18 chromosome number, *A. wilhelmsii* and *A. vermicularis* showed 2n = 4x = 36 and *A. millefolium* showed 2n = 6x = 54 chromosome number. The chromosome numbers of *A. eriophora* and *A. talagonica* are new to science and new polyploidy levels are reported for *A. tenuifolia* and *A. wilhelmsii*. Tetraploid and hexaploid species, they formed only bivalents in metaphase of meiosis-I showing diplontic behavior possibly due to allopolyploid nature of the species studied and the presence of control over pairing among homologous chromosomes. Multipolar cells were observed almost in all populations and species studied leading to the formation of abnormal tetrads and pollen grains as well as unreduced (2n) pollen formation.
Acta Biol Szeged 53(1):39-43 (2009)

KEY WORDS

Achillea sp.
chromosome numnber
cytology
pollen formation

The genus *Achillea* L. (Asteraceae) has approximately 130 perennial herb species (Saukel et al. 2004; Guo et al. 2004, 2005), mostly distributed in Eurasia, some in North Africa, while a few species can be found in North America and in the Southern Hemisphere (Bremer and Humphries 1993). A large number of species are endemic and restricted to certain regions, in contrast to other species from the genus growing over a wide geographical range.

Members of *Achillea* L. are usually herbaceous perennials, entomophilous and predominantly outbreeding, the basic chromosome number is x = 9 with most of the species being diploid. Polyploid taxa have originated in many clades including 4x, 6x and 8x species. The genus exhibits great ecological amplitude ranging from deserts to water-logged habitats and from sea coasts to the high mountains (Ehrendorfer and Guo 2006), as a result of which, several *Achillea* species show high morphological variability. For example, the unramnified forms of *A. millefolium* L. can easily be mistaken for *A. collina* Becker (Dabrovska 1977) under arid conditions; the leaf dissection of populations belonging to the *A. millefolium* complex differs dramatically along an altitudinal gradient in the Sierra Nevada due to both genetic and other components (Gurevitch 1988). Biste (1978) described considerable variations in height, leaf width, shoot number, branching and stomata length in populations of different origin of the same species.

The majority of the *Achillea* species are of medicinal values having therapeutic applications. The whole ground parts and mainly the inflorescences are effective as anti-inflammatory, spasmolytic, choleretic drugs. Essential oil and extracts of the plants are used for preparation of cosmetics, stomachic and digestive teas, creams, etc. Besides 1,8-cineole, compounds of bornane skeleton such as camphor and borneol are among the second and third most frequently characterized components of yarrow oil in *A. taygetea* and *A. fraasi* (Magiatis et al. 2002), *A. albicaulis* C. A. Mey. (Feizbakhs et al. 2003), *A. pseudoaleppica* Hub.-Mor. (Zen et al. 2003), *A. pachycephala* Rech.f. (Bamasian et al. 2002), *A. talagonica* Boiss. and *A. vermicularis* Trin (Rustaiyan et al. 1998).

Nineteen *Achillea* species are reported from Iran (Podlech 1986) growing in different regions of the country. Although *Achillea* species have been studies extensively in different regions of the world (Dabrovska 1977; Pireh and Tyrl 1980; Dabrowska 1989; Lambrou et al. 2004; Guo et al. 2004; Saukel et al. 2004; Guo et al. 2005; Ahmet 2006; Yasar et al. 2008), similar studies are almost completely lacking in Iran. Therefore the present study considers cytogenetic characteristics of 14 populations belonging to 8 *Achillea* species growing in Iran, reporting new chromosome number as well as the occurrence of B-chromosomes and unreduced gamete (2n) formation in some of the species for the first time.

Materials and Methods

Cytological studies were performed in 14 populations of 8 *Achillea* species growing in Iran namely 1- *Achillea biebersteinii* Afan., 2- *A. oxyodonta* Boiss. (two populations), from the sect. *Flipendulinae*, 3- *A. millefolium* L., from the sect.

Accepted May 4, 2009
*Corresponding author. E-mail: msheidai@yahoo.com

Figure 1. Representative meiotic cells in the *Achillea* species studied. A-D = Meiocyte in *A. eriophora, A. biebersteinii, A. oxyodonta* and *A. talagonica* showing 2n = 18 chromosome number. E = Meiocyte in *A. tenuifolia* showing 2n = 18 and heterozygote translocation (small arrow) as well as B-chromosome (big arrow). F = Meiocyte showing 2n = 38 and one quadrivalent in Polour population of *A. vermicularis*. G & H = Meiocytes showing 2n = 38 in Abali and Quem populations of *A. wilhelmsii*. I = Meiocyte showing chromosome stickiness in Touchal population of *A. oxyodonta*. J = Meiocyte showing chromosome clumping in *A. eriophora*. K = Meiocyte showing micronucleus in (arrow) in Quem population of *A. wilhelmsii*. L & M = Tripolar and multipolar cells in *A. wilhelmsii*. N – P = Bigger pollen (potential 2n) grains in Chitgar population of *A. wilhelmsii*, Polour population of *A. vermicularis* and Touchal population of *A. oxyodonta* respectively. Scale bar = 10 μm.

Millefolium, 4- *A. tenuifolia* Lam., 5- *A. vermicularis* Trin. (two populations), 6- *A. wilhelmsii* K. Koch. (five populations), 7- *A. eriophora* DC., Prodr., and 8- *A. talagonia* Boiss., from the sect. *Santolinoidea*. The voucher specimens are deposited in Tehran University Herbarium and Shahid Beheshti University Herbarium (SBUH).

Meiotic studies were performed on young flower buds collected using minimum 100 metaphase/ diakinesis pollen mother cells (PMCs) and 500 anaphase and telophase cells for data collection (Sheidai and Rashid 2007). Pollen satiability as a measure of fertility was determined by staining minimum 1000 pollen grains with 2% acetocarmine: 50% glycerin (1:1) for about ½ hr. Round. Complete pollens which were stained were taken as fertile, while incomplete, shrunken pollens with no stain were considered as infertile (Sheidai and Rashid 2007).

χ^2 test was performed to detect a significant difference in chiasma frequency and chromosome pairing as well as meiotic abnormalities (Sheidai and Rashid 2007). Similar test was performed among different species and populations having different chromosome numbers by using relative meiotic data. For this purpose the relative chiasma frequency was determined by dividing chiasma frequency and also the number of ring and rod bivalents by the haploid chromosome number (Sheidai and Bagheri-Shabestarei 2007).

In order to detect significant difference between potential unreduced pollen grains and the normal (reduced pollens), t-test was performed. Cytogenetic similarities and distinctness of the species was studied by using different clustering and ordination methods (Sheidai and Bagheri-Shabestarei 2007). Statistical analyses used SPSS ver. 9 (1998) and DARwin ver. 5.0.155 (2006) software.

Results and Discussion

Chromosome pairing and segregation

Data with regard to chiasma frequency and distribution as well as chromosome pairing are provided in Table 1, Figures 1-4. *A. eriophora*, *A. tenuifolia*, *A. oxyodonta*, *A. talagonica* and *A. biebersteinii* showed 2n = 2x = 18 chromosome number, *A. wilhelmsii* and *A. vermicularis* showed 2n = 4x = 36 and *A. millefolium* showed 2n = 6x = 54 chromosome number (Fig. 1A-H).

The chromosome numbers obtained for *A. biebersteinii*, *A. millefolium*, *A. vermicularis* and *A. wilhelmsii* supports the earlier reports (Morton 1981; Dabrowska 1989; Khaniki 1995; Sulborska 2006), while the chromosome numbers of *A. eriophora* and *A. talagonica* are new to science. Khaniki (1995) reported 2n = 3x = 27 for *A. tenuifolia* and 2n = 18 for *A. wilhelmsii* from Iran while, we report 2n = 2x = 18 and 2n = 36 for these species respectively. Therefore two different polyploidy levels exist for these species. Similar situation is known to occur in other *Achillea* species like *A. millefolium* (2n= 36 and 2n= 45, Gervais 1977; Khaniki 1995) and *A. vermicularis* (2n = 18 and 2n= 36, Dabrowska 1989; Khaniki 1995), indicating the role played by polyploidy in the species diversification of *Achillea*.

Among three populations of *A. wilhelmsii* studied, the highest value of total and terminal chiasmata occurred in Karaj and Quem populations (24.90 & 242.81 respectively), while the lowest value of the same occurred in Damavand and Abali populations (20.70 & 13.00 respectively). The highest value of intercalary chiasmata occurred in Abali population (9.67) and the lowest value of the same occurred in Karaj population (2.16).

Chitgar-Park and Abali populations of *A. wilhelmsii* showed the highest mean value of ring bivalents (11.00), while Damavan population showed the highest mean value of rod bivalents (12.85).

Some of tetraploid and hexaploid *Achillea* species and populatins showed diplontic behavior and formed only bivalents in metaphse of meiosis-I (Fig. 1A-D, G & H), while

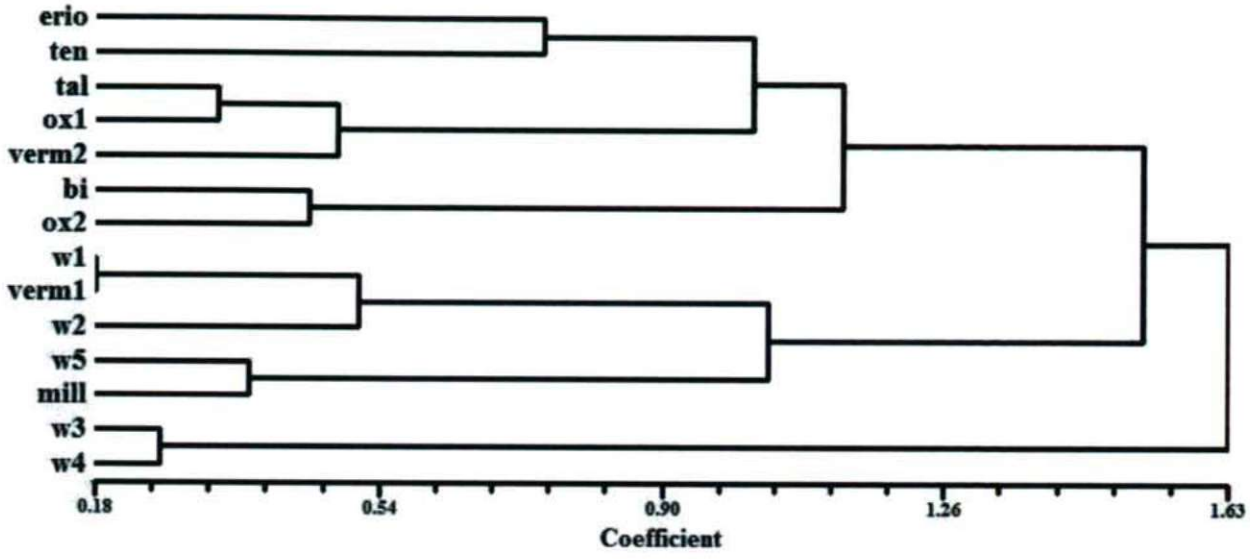


Figure 2. UPGMA dendrogram of *Achillea* species studied. Species abbreviations: erio = *A. eriophora*, ten = *A. tenuifolia*, tal = *A. talagonia*, ox1 & ox2 = Touchal and Darbandsar populations of *A. oxyodonta* respectively, verm1 & 2 = Polour and Darbandsar populations of *A. vermicularis* respectively, bi = *A. biebersteinii*, mill = *A. millefolium* and w1-5 = Quem, Karaj, Chitgar, Damavand and Abali populations of *A. wilhelmsii* respectively.

Damavan and Polour populations of *A. wilhelmsii* and *A. vermicularis* formed very low amount of quadrivalents (Fig. 1F). Such diplontic behavior may be due to allopolyploid nature of the species studied and the presence of control over pairing among homologous chromosomes also suggested in other *Achillea* species (Ehrendorfer 1959; Loidi et al. 1990).

Cytogenetic studies in hybrid *Achillea* plants (Ehrendorfer 1959; Loidi et al. 1990) showed that chromosome pairing occurrs among homologous chromosomes only and not among homoeologous chromosomes of the parental genomes. For example in a pentaploid hybrid between *A. collina* (4x) and *A. millefolium* (6x), the five corresponding chromosomes of each set were assorted into a group of three (two synapsed plus one aligned) and a group of two (synapsed), reflecting different degrees of homology between the five genomes. The pentaploid hybrid received three homoeologues from its hexaploid parent (*A. millefoliurn*) and two homoeologues from its tetraploid parent (*A. collina*), pairing between the parental homoeologous groups seemed to be largely suppressed (Ehrendorfer 1959).

χ^2 test did not showed a significant difference for relative chiasma frequency and chromosome pairing among *Achillea* species and populatins studied indicating that no significant change has occurred in the number genes controlling chromosome pairing.

Although *A. tenuifolia* studied is diploid and is expected to form only bivalents in metaphse of meiosis-I, very low amount of quadrivalents were observed (Fig. 1E), indicating the occurrence of heterozygote translocations between two pairs of chromosomes. Such chromsomal structural changes may increase the amount of genetic variability in the gametes by forming new genetic linkage groups which may be used for adaptation to adverse environmental conditions.

Variation in chiasma frequency and localization is genetically controlled (Quicke 1993) and has been reported in populations of different species (Rees and Jones 1977). Such a variation in the species and populations with the same chromosome number is considered as a means for generating new forms of recombination influencing the variability within natural populations in an adaptive way (Rees and Jones 1977).

Several studies show the importance of hybridization along with polyploidy in the evolution of *Achillea* (Ehrendorfer 1959; Uotila 1979). For example in the group *Millefolium* having the most important medicinal species of the genus, in addition to diploid to the octoploid sppecies available, aneuploids also often occur presumably as a result of interspecific hybridization (Uotila 1979).

Grouping of the species based on UPGMA (Unweighted Paired group with Arithmetic Average) and NJ (Neighbor joining) clustering as well as ordinaton plots based on PCO (Principal Coordinate Analysis) and PCA (Principal Component Analysis) produced similar results (Figs. 2 & 3), almost separating the species of the three sections studied from each other and indicating their cytogenetic distinctness.

laggard chromosomes and chromosomes stickiness were observed during anaphase I and II as well as telophase-I and II (Fig. 1I) in most of the species and populations studied.

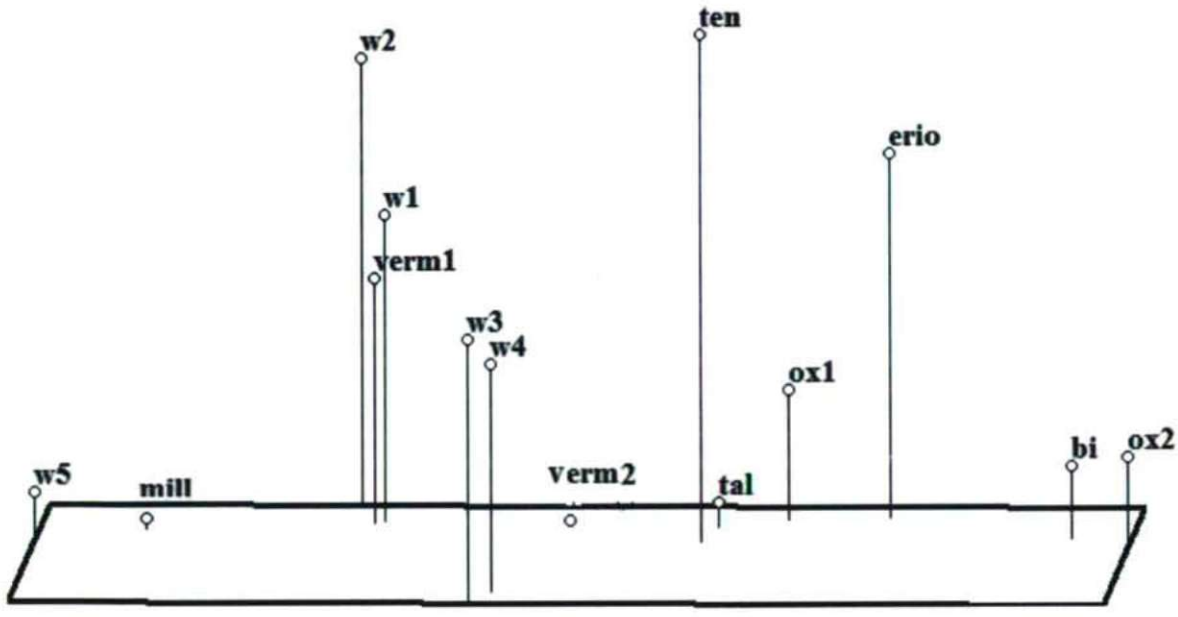


Figure 3. PCO plot of *Achillea* species studied. Species abbreviations: erio = *A. eriophora*, ten = *A. tenuifolia*, tal = *A. talagonia*, ox1 & ox2 = Touchal and Darbandsar populations of *A. oxyodonta* respectively, verm1 & 2 = Polour and Darbandsar populations of *A. vermicularis* respectively, bi = *A. biebersteinii*, mill = *A. millefolium* and w1-5 = Quem, Karaj, Chitgar, Damavand and Abali populations of *A. wilhelmsii* respectively.

The highest percentage of anaphase-I laggards occurred in Darbandsar population of *A. vermicularis* (3.20), while the highest value of the anaphase-II laggards occurred in Karaj population of *A. wilhelmsii* (1.80). The lowest value of cells showing telophase-I laggards occurred in Darbandsar population of *A. oxyodonta* (1.70%), while the highest value of the same occurred in Abali population *A. wilhelmsii* (Fig. 1I). Laggards observed may be the reason for micronuclus formation observed in some of the species studied (Fig. 1K).

The sticky chromosomes occurred from early stages of prophase to the final stages of meiosis. The number of chromosomes involved in stickiness varied from two to many forming a complete clumping of the chromosomes (Fig. 1J). χ^2 test showed a significant difference for the percentage of chromosome stickiness and laggards among the species and populations studied. Genetic and environmental factors as well as genomic-environmental interaction have been considered as the reason for chromosome stickiness in different plant species (Nirmala and Rao 1996; Baptista-Giacomelli et al. 2000).

Multipolar cells were observed (Fig. 1L & M) almost in all populations and species studied which may be due to spindle abnormalities. Such meiotic abnormalities may lead to the formation of abnormal tetrads and pollen grains, the occurrence of aneuploidy condition as well as unreduced (2n) pollen formation (Villeux 1985; Nirmala and Rao 1996). The *Achillea* species studied showed pollen fertility percentage of 77.00-99.00, meiotic abnormalities observed may be responsible for pollen sterility observed.

Unreduced pollen grain formation

The occurrence of large pollen grains (possibly 2n pollen grains) was observed along with smaller (normal) pollen grains almost in all species and populations studied (Fig. 1N-P). The large pollen grains comprised about 1.00-3.30% of pollen grains in these populations.

The mean diameter of normal (reduced) pollen grains ranged from 16.15 µm to 23.00 µm while, the mean diameter of unreduced pollen grains ranged from 28.10 µm to 38.90 µm. T-test analysis revealed a significant difference ($p < 0.001$) for the size between the larger sized pollen grains and smaller sized pollen grains. The presence of giant pollen grains has been used as an indication of the production of 2n pollen (Vorsa and Bingham 1979; Bertagnolle and Thomson 1995). Ramsey (2007) also reported the occurrence of unreduced pollen grains in *A. borealis* with the mean values of 19.00 µm for normal (reduced) pollen grains and 27.50 µm for unreduced pollen grains, well in the range we observed for other *Achillea* species.

Unreduced gametes are known to produce individuals with higher ploidy level through a process known as sexual polyploidization (Villeux 1985), which has been considered as the major route to the formation of naturally occurring polyploids. Different cytological mechanisms are responsible for the production of 2n gametes (Bertagnolle and Thomson 1995). The occurrence of multipolar cells and irregularities in anaphase segregation of the chromosomes might be considered as the possible mechanisms of unreduced pollen grain

formation in the *Achillea* species and populations studied. The occurrence of unreduced pollen grains was previously reported only in *A. borealis* (Ramsey 2007), therefore this is the first report on the occurrence of unreduced pollen grains in the other species of the *Achillea*.

B-chromosomes

B-chromosomes (Bs) of 0-1 were observed in *A. tenuifolia*. The Bs observed were much smaller than the A-chromosomes, round in shape and did not pair with the A-chromosomes. B-chromosomes are accessory chromosomes occurring in more than 1300 species of plants and almost 500 species of animals (Camacho et al. 2000). B-chromosomes have been reported in some other *Achilleae* species like *A. distans* (Dabrowska 1992), *A. glaberrima* (Dabrowska 1989), *A. lingulata* (Dabrowska 1989) and *A. nobilis* (Siljak-Yakovlev 1982). The B-chromosomes when present in high number affect negatively the growth and vigor of the plants, while in low number may benefit the plant possessing them.

References

Ahmet S (2006) Karyological notes on eight species of *Achillea* L. (Asteraceae, Santolinoideae) from Turkey. Bot J Lin Soci 151:573-580.

Bamasian S, Rustaiyan A, Masoudi S (2002) Chemical constituents of the essential oil of *Achillea pachycephala* Rech.f., a species endemic to Iran. 33rd International Symposium on Essential Oils, Abstracts, p. 50.

Baptista-Giacomelli FR, Pagliarini MS, Almeida JL (2000) Meiotic behavior in several Brazilian oat cultivars (*Avena sativa* L.). Cytologia 65:371-378.

Bertagnolle F, Thomson JD (1995) Gametes with the somatic chromosome number: Mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol 129:1-22.

Biste C (1978) Cytotaxonomische Untersuchungen des Formenkreises *Achillea millefolium* (Asteraceae) in der DDR. Fed Report 88:533-613.

Bremer K, Haumphries CJ. 1993. Genetic monograph of the Asteraceae-Anthemideae. Bull Nat Hist Mus Lond (Bot) 23:71-177.

Camacho JPM, Sharbel TF, Beukeboom LW (2000) B-chromosome evolution. Phil Trans R Soc Lond B 355:163-178.

Dabrovska J (1977) Observations of the fruit size of nine taxons from the genus *Achillea* L. representing natural di-, tetra-, hexa- and octaploids. Herba PoL 28:55-66.

Dabrowska J (1989) The chromosome numbers of several taxa of the genus *Achillea* L. Acta Soci Bot Pol 58:163-177.

Dabrowska J (1992) Chromosome number and DNA content in taxa of *Achillea* L. in relation to the distribution of the genus Prace Bot 49:1-83.

Ehrendorfer F (1959) Differentiation-hybridization cycles and polyploidy in *Achillea*. Cold Spring Harbor Symp quant Biol 24:141-152.

Ehrendorfer F, Guo YP (2006) Multidisciplinary studies on *Achillea* sensu lato (Compositae-Anthemideae): new data on systematics and phylogeography Willdenowia 36:69-87.

Feizbakhs A, Tehrani MS, Rustaiyan A (2003) Composition of the essential oil of *Achillea albicaulis* CA Mey. J Essent Oil Res 15:21-22.

Guo YP, Ehrendorfer F, Samuel R (2004) Phylogeny and systematics of *Achillea* (Asteraceae-Anthemideae) inferred from nrITS and plastid *trnL-F* DNA sequences. Taxon 53:657-672.

Guo YP, Saukel J, Mittermayr R, Ehrendorfer F (2005) AFLP analyses demonstrate genetic divergence, hybridization, and multiple polyploidization in the evolution of *Achillea* (*Asteraceae-Anthemideae*). New Phytol 166:273-289.

Gervais C (1977) Cytological investigation of the *Achillea millefolium* complex (Compositae) in Quebec. Can J Bot 55:796-808.

Gurevitch J (1988) Variation in leaf dissection and leaf energy budgets among populations of Achillea from an altitudinal gradient. Amer J Bot 9:1298-1306.

Khaniki GB (1995) Chromosome numbers and morphometry in *Achillea* (Anthemideae, Compositae). Nucleus 38:104-111.

Loidi J, Ehrendorfer F, Schweizer D (1990) EM analysis of meiotic chromosome pairing in a pentaploid Achillea hybrid. Heredity 65:11-20.

Magiatis P, Skaltsounis AL, Chinou I, Haroutounian SA (2002) Chemical composition and in vitro antimicrobial activity of the essential oils of the greek Achillea species. Z Natur 57:287-290.

Morton JK (1981) Chromosome numbers in Compositae from Canada and the U.S.A. Bot J Lin Soci 82:357-368.

Nirmala A, Rao PN (1996) Genetics of chromosome numerical mosaism in higher plants. Nucleus 39:151-175.

Pireh W, Tyrl RJ (1980) Cytogeography of *Achillea millefolium* in Oklahoma and adjacent States. Rhodora 80: 361-367.

Podlech D (1986) Compositeae VI. Antenideae. In: Flora Iranica, ed. Rechinger K. H., NO. 158., pp 49-71. Skademische Druck-u., Verlagsans Talt, Graz, Austria.

Quicke DLJ (1993) Principles and techniques of contemporary taxonomy. Blackie Academic & Professional, Glasgow.

Rustaiyan A, Komeilizadeh H, Shariatpanahi MS, Jassbi A, Masoudi S (1998). Comparative study on the essential oils of three *Achillea* species from Iran. J Essent Oil Res 10:207-209.

Ramsey J (2007) Unreduced gametes and neopolyploids in natural populations of *Achillea borealis* (Asteraceae). Heredity 98:143-150.

Rees H, Jones RN (1977) Chromosome Genetics. London, Edward Arnold.

Romero-Zarco C (1986) A new method for estimating karyotype asymmetry. Taxon 35:526-530.

Saukel J, Anchev M, Guo YP, Vitkova A, Nedelcheva A, Goranova A, Konakchiev A, Lambrou M, Nejati S, Rauchensteiner F, Ehrendorfer F (2004) Comments on the biosystematics of *Achillea (Asteraceae-Anthemideae)* in Bulgaria. Phytol Balcanica 9:361-400.

Sheidai M, Bagheri-Shabestarei ES (2007) Cytomixis and unreduced pollen formation in some *Festuca* L. species of Iran. Caryologia 60:364-371.

Sheidai M, Rashid S (2007) Cytogenetic study of some *Hordeum* L. species in Iran. Acta Biol Zseged 51:107-112.

Siljak-Yakovlev S (1982) In IOPB chromosome number reports LXXVII. Taxon 31:768

Sulborska A (2006) Morphology, anatomy and ultrastructure of yarrow (*Achillea millefolium* L.) floral nectaries Acta Agrobot 59:17-28.

Uotila P (1979) Hybridization of *Achillea salicifolia* and *A. ptarmica* (Compositae). Ann Bot Fenn 16:374-382.

Villeux R (1985) Diploid and polyploid gametes in Crop Plants: Mechanisms of formation and utilization in plant breeding. In: JANICK, J., (ed.), Plant Breed. Rev., Vol. 3., AVI Publishing Co. Wesport, Connecticut, 442 p.

Yasar K, Arabaci T, Sahin A, Turkoglu I (2008) Karyological notes on another eight species of Achillea (Asteraceae) from Turkey. Biologia 63:343-348.

Zen HC, Toker Z, Clery RA, Owen NE (2003) Composition of the essential oil of *Achillea pseudoaleppica*. Hub-Mor. J Essent Oil Res 15:96-97.

Volume 53(1):45-49, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Antimicrobial activity of *Acorus calamus* (L.) rhizome and leaf extract

Asha Devi S and Deepak Ganjewala*

School of Biotechnology, Chemical and Biomedical Engineering, Vellore Institute of Technology University, Vellore-632 014 (T.N.), India

ABSTRACT Antimicrobial activity of *Acorus calamus* rhizome and leaf extracts obtained with different solvents *viz.*, petroleum ether, chloroform, hexane and ethyl acetate was evaluated. Extracts obtained with ethyl acetate among others were found to be highly effective. Rhizomes and leaf ethyl acetate extracts exhibited pronounced antifungal activity with diameter zone of inhibition ranged from 20-28 and 18-25 mm as well as antiyeast activity with diameter zone of inhibition ranged from 22-25 and 20-23 mm, respectively. The minimum inhibitory concentration (MIC) of the rhizome and leaf extracts for antifungal activity measured was 2-4mg/ml, except *Penicillium chrysogenum* whereas against yeasts was relatively higher, 4-5 and 6-8 mg/ml. MIC value for antibacterial activity was comparatively very high ~16-42 mg/ml. In addition, authentic α- and β-asarones were also tested for their antimicrobial potential. Both α- and β-asarones exhibited very strong antimicrobial activities against the fungi and yeasts than those of rhizome and leaf extracts. The study clearly suggested that *A. calamus* rhizomes and leaves must possess active principle α- and β-asarones which is believed to be responsible for their antimicrobial activities. Both rhizomes and leaf extracts, however, had no antibacterial activity except *E. coli*.
Acta Biol Szeged 53(1):45-49 (2009)

KEY WORDS

Acorus calamus
antimicrobial, asarone
minimum inhibitory concentration
zone of inhibition

Our interest in plants because of their medicinally and pharmacologically important active ingredients is increasing rapidly. Plant produces a plethora of natural products, such as alkaloids, phenolics, flavonoids and terpenoids or isoprenoids which has often been correlated with medicinal and pharmacological properties of the plants. These plant natural products have been studied extensively and their role in several useful biological activities, such as antibacterial, antifungal, antiyeast, insecticides and herbicides have been well documented. Number of plant products with the useful bioactive properties is increasing rapidly as an outcome of several ongoing research programs on investigation of biological activities of a number of plants. These bioactive compounds have served as lead molecules for the development of many synthetic potential antibiotics. *Acorus calamus* (L.) family Araceae is a well known plant in Indian traditional medicines (Mehrotra et al. 2003) for centuries. It is a perennial, semi-aquatic and smelly plant, found in both temperate and sub temperate zones. It grows up to 6 feet tall with sword-shaped leaves, small yellow/green flowers and branched rhizomes (Sabitha et al. 2000). The rhizomes, roots and essential oil distilled from these plant parts have been reported to posses several important biological activities including antifungal (Lee et al. 2004; Lee et al. 2005), antibacterial (McGraw et al. 2002; Phongpaichit et al. 2005), allelopathic (Nawamaki and Kuroyanagi 1996), anticellular and immunosuppressive (Mehrotra et al. 2003). Essential oil of *A. calamus* possesses antigonadal activity in insects (Mathur and Saxena, 1975; Koul et al. 1977a, b; Saxena et al. 1977; Schmidt and Brochers 1981). Aromatic oils obtained by alcoholic extraction of the rhizome are used in the pharmaceutical and oenological industries (Bertea et al. 2005). *A. calamus* whole plant and plant parts *viz.*, roots, rhizomes, leaves and essential oil have been extensively investigated for chemical compositions, previously (Namba 1993; Wang et al 1998; Raina et al. 2003; Venskutonis and Dagilyte 2003). Previous studies on chemical investigations of *A. calamus* have revealed the presence of various compounds, such as asarone (α- and β), caryophyllene, isoasarone, methyl isoeugenol and safrol in rhizome and roots (Namba 1993; Wang et al. 1998). Essential oils also have a very similar chemical composition to that of rhizomes, dominated by α- and β-asarone (Lander and Schreier 1990; Oprean et al. 1998; Raina et al. 2003; Venskutonis and Dagilyte 2003). Both α- and β- asarone identified as the major chemical constituent in roots, rhizomes, leaves and essential oils held responsible for almost all the biological activities of *A. calamus*. Despite the fact that *A. calamus* roots, rhizomes and essential oils possess several useful biological activities, sufficiently not enough efforts have been devoted to study their antifungal and antiyeast properties. In the present study we investigated antimicrobial activities of rhizomes and leaf

Accepted April 22, 2009
*Corresponding author. E-mail: deepakganjawala73@yahoo.com

extracts against fungi, yeasts and bacteria (gram negative and positive). Simultaneously, we investigated antimicrobial activities of the active principles α- and β-asarones. The present clearly has suggested that the active principle α- and β-asarones are responsible for the antimicrobial activity of rhizome and leaf extracts.

Materials and methods

Plant materials

Plants were collected from Horticultural Research Station, Yercaud in Tamil Nadu, India and grown at Herbal Garden, Vellore Institute of Technology University, Vellore, India. The fresh rhizomes and leaves were collected, washed thoroughly. A known amount of rhizome and leaves (100 g each) were kept in an oven at 40°C for drying till constant weight. Rhizome and leaves thus dried were powdered in a mixer grinder.

Extract preparation

A known amount of leaf and rhizome powder (4 g each) was extracted separately with different solvents viz., petroleum ether, chloroform, hexane and ethyl acetate for 24 hrs as per the procedure published previously (Mehrotra et al. 2003). Extracts thus prepared were weighed and stored at 4°C.

TLC analysis

The extracts were subjected to thin layer chromatographic separation. 5-10 µL of extracts were loaded on pre-coated silica gel-G plates along with authentic standard α- and β-asarone (Sigma Aldarich). The plates were developed in a chamber saturated with solvent system toluene: ethyl acetate (v/v, 8:2) described by Oprean et al. (1998). The plates were removed from the chamber, dried and observed under visible and UV light for visualization of the spots. Presence or absence of the α- and β-asarone in the extracts was confirmed by authentic α- and β-asarone.

Antimicrobial screening

Microorganisms

All the microorganisms except *Vibrio cholera* (Clinical isolates) used in the present study were obtained form Microbial Type Culture Collection (MTTC), Institute of Microbial Technology, Chandigarh and National Collection of Industrial Microorganisms (NCIM), Pune, India. Microorganisms used includes bacteria viz., *Escherichia coli* MTCC901, *E. coli* NCIM, *Pseudomonas aeruginosa* MTCC429, *Salmonella parathypi* A MTCC735, *Enterococcus faecalis* MTCC 439, *Staphylococcus aureus* MTCC96 , *Shigella sonnei* MTCC2957fungi viz., *Penicillium chrysogenum* MTCC2725, *Aspergillus niger* MTCC1344, *A. Flavus* MTCC2799, *Microsporum canis* MTCC2820 and yeasts viz., *Cryptococcus gastricus* MTCC1715, *Candida albicans* MTCC3017, *C. albicans* NCIM. Bacterial cultures were maintained in the nutrient agar medium, fungi and yeast in Sabourd Dextrose agar (SDA)

Antibacterial activity

Antibacterial activities of rhizome and leaf extracts were evaluated by agar well diffusion method (Priya and Ganjewala 2007). Simultaneously, antimicrobial activity of α and β-asarones were also investigated. 24 h broth cultures of the bacteria used for the antibacterial assay. A sterile cotton swab dipped into bacterial suspension and evenly streaked over the entire surface of sterile Muller Hinton agar plate to obtain uniform inoculum. Wells were punched on the seeded plates using sterile borer (8 mm). 20 to 200 µL of rhizome and leaf extracts obtained with ethyl acetate, petroleum ether, chloroform and hexane (100 mg extract dissolved in 1 ml of dimethyl sulfoxide, DMSO) were dispensed into each well using sterile micropipette. Streptomycin (30µg/ml) was used as a positive control. The plates were then incubated at 37°C for 18 h and diameter zone of inhibition was measured. The minimum inhibitory concentration (MIC) values were determined by double dilution method (Lorian 1996). The rhizome and leaf extract as well as both α- and β-asarone were serially diluted in double strength of Muller Hinton broth. The broths then inoculated with 100 µl of bacterial culture. The MIC is the highest dilution of extract which shows clear fluid with no developments of turbidity. The absence of growth was confirmed again on solid media. In the second set of experiment α and β-asarone (10 mg/ml DMSO) were used for the antibacterial screening in similar way.

Antifungal and Antiyeast activity

Antifungal and antiyeast activity assay was performed using well diffusion method. 20-200 µL of the extracts 100 mg/ml DMSO was loaded into wells in SDA plates. Simultaneously, amphotericin B (50 µg/ml) was also loaded as a positive control. The plates were then incubated at room temperature for 2-4 days and diameter zone of inhibition was measured. The MIC values were determined for rhizome and leaves extract as well as both α- and β-asarone by following previous method using Sabourd dextrose broth.

Results

Antimicrobial activities of rhizome and leaves extracts

Our preliminary study of antibacterial assay revealed that only ethyl acetate extracts of rhizome and leaf exhibited strong inhibitory action against the microorganisms tested, however, extracts in other solvents did not show any activities. Antimicrobial potentials of the extracts were evaluated

Table 1. Antimicrobial activity of rhizome and leaf ethyl acetate extracts. 200µL extracts (100mg/ml DMSO) were used in the antimicrobial screening.

Microorganism	Zone of inhibition (mm)			MIC (mg/ml)	
	Rhizome	Leaves	Streptomycin (30µg/ml)	Rhizome	Leaves
Bacteria (Gram negative)					
Escherichia coli MTCC901	20	18	21	16	18
Escherichia coli NCIM	25	22	21	42	42
Pseudomonas aeruginosa MTCC 429	R	R	17	ND	ND
Salmonella parathypi A MTCC 735	R	R	22	ND	ND
Shigella sonnei MTCC2957	R	R	18	ND	ND
Vibrio cholera	R	R	18	ND	ND
Bacteria (Gram positive)					
Enterococcus faecalis MTCC 439	R	R	15	ND	ND
Staphylococcus aureus MTCC 96	R	R	16	ND	ND
Fungi			Amphotericin B (50µg/ml)		
Penicillium chrysogenum MTCC2725	22	20	18	32	32
Aspergillus niger MTCC 1344	25	20	21	2	2
Aspergillus Flavus MTCC2799	28	25	18	3	4
Microsporum canis MTCC 2820	20	18	19	4	4
Yeast					
Cryptococcus gastricus MTCC1715	22	20	18	5	6
Candida albicans MTCC 3017	25	23	22	4	8
Candida albicans NCIM	23	22	21	4	8

R = resistant; ND = Not determined

by measuring the diameter zones of inhibition (mm) as well as minimum inhibitory concentration (MIC). Further, the study revealed that ethyl acetate extract of rhizome and leaves exhibited strong activity against fungi and yeasts but not the bacteria tested (Table 1). Only *Escherichia coli* (MTCC901 and NCIM) was found to be highly sensitive to both rhizomes and leaves extracts. Rest of the bacteria gram negative as well as positive was found to be resistant to rhizome and leaves extract. Rhizome and leaves extract showed marked antifungal activity against *Aspergillus niger* (MTCC 1344), *A. Flavus* (MTCC2799), *Microsporum canis* (MTCC 2820) except *Penicillium chrysogenum*, (MTCC2725) with zone of inhibition (ZI) ranged 20-28 and 18-25 mm, respectively along with MIC value 2-4 mg/ml for each rhizome and leaves extract. *Aspergillus flavus* among other was found to be most sensitive both to the rhizome and leaf ethyl acetate extract (Table 1). Rhizome and leaf extracts also displayed significant antiyeast activity against *Cryptococcus gastricus* (MTCC1715), *Candida albicans* (MTCC 3017 and NCIM) with diameter zone of inhibition 22-25 and 20-23 mm, respectively along with MIC value ranged 4-5 mg/ml for rhizome extract and 6-8 mg/ml for leaves extract. Both rhizome and leaf extracts were not effective against bacteria tested except *E. coli*. however rhizome (MIC 16-42 mg/ml) and leaf extracts (18-42 mg/ml) has shown considerable antibacterial activity against *Escherichia coli* (MTCC901 and NCIM) with dimeter zone of inhibition 20-25 and 18-22 mm, respectively.

Antimicrobial activities of the α and β-asarone

Both α- and β-asarone exhibited significant and comparable antimicrobial activities (zone of inhibition) to that of rhizome and leaf extracts but with relatively less MIC values (Table 2). Overall the study showed that β- asarone had relatively higher antimicrobial activity than the α-asarone. For instance, β-asarone displayed with MIC value 0.5-8 mg/ml a high antifungal activity (ZI, 20-29 mm) whereas α-asarone displayed almost equal antifungal activity (ZI, 19-27 mm) but with high MIC value 2-8 mg/ml. Trends of antiyeast activity obtained for α- and β-asarone was almost similar to that of antifungal activity except slight alteration in the ZI and MIC values. Both α- and β-asarone did not display antibacterial activity except *E. coli* (MTCC901 and NCIM).

Discussion

Several previous and recent studies have described many important biological activities, particularly antimicrobial of *A. calamus* roots, rhizome and essential oils (Grosvenor et al. 1995; Mungkornasawakul 2000; MacGaw et al. 2002; Rani et al. 2003; Phongpaichit et al. 2005). Although our present study only further strengthened and supported previously published reports on useful biological properties of the *A. calamus*, for the first time we attempted to investigate antimicrobial properties of the leaves derived active ingredients. Overall analysis of the results obtained here has clearly

Table 2. Antimicrobial activity of α-and β-asarone. 200 µL of α and β-asarone (10 mg/ml DMSO) were used in the antimicrobial screening.

Microorganism	Zone of inhibition (mm)			MIC(mg/ml)	
	α-asarone	β-asarone	Streptomycin (30µg/ml)	α-asarone	β-asarone
Bacteria (Gram negative)					
Escherichia coli MTCC901	19	22	21	16	8
Escherichia coli NCIM	20	24	21	32	16
Pseudomonas aeruginosa MTCC 429	R	R	17	ND	ND
Salmonella parathypi A MTCC 735	R	R	22	ND	ND
Shigella sonnei MTCC2957	R	R	18	ND	ND
Vibrio cholera	R	R	18	ND	ND
Bacteria (Gram positive)					
Enterococcus faecalis MTCC 439	R	R	15	ND	ND
Staphylococcus aureus MTCC 96	R	R	16	ND	ND
Fungi			Amphotericin B (50 µg/ml)		
Penicillium chrysogenum MTCC2725	24	22	18	8	4
Aspergillus niger MTCC 1344	22	26	21	2	0.5
Aspergillus Flavis MTCC2799	27	29	18	3	0.5
Microsporum canis MTCC 2820	19	20	19	4	8
Yeast					
Cryptococcus gastricus MTCC1715	24	26	18	5	6
Candida albicans MTCC 3017	26	28	22	2	0.5
Candida albicans NCIM	22	23	21	2	0.5

R = resistant; ND = Not determined

showed that rhizome predominantly possess bioactivities (antifungal and antiyeast) than any other plant parts such as leaf that has less bioactive effects (Fig. 1). Although both rhizome and leaf extracts demonstrated substantial antifungal and antiyeast activities, they did not show any antibacterial activity except that of *E. coli*. Previously, De et al. (1999) in his report on antimicrobial activities of *A. calamus* has described lack of antibacterial activity while recently Phongpaichit et al. (2005) have observe very less antibacterial activity in his study on antimicrobial properties of *A. calamus* rhizome. Our results of lack of antibacterial activities hence are in full agreement with those reported by De et al. (1999) and Phongpaichit et al. (2005). Although, there are several published reports are also available on antibacterial activity of *A. calamus extracts* (Grosvenor et al. 1995; MacGaw et al. 2002; Rani et al. 2003). A recent study by Phongpaichit et al. (2005) has revealed strong antimicrobial (antifungal and antiyeast) properties of the crude methanol extracts of *A. calamus rhizome*. In their study they found that methanol extract of the rhizome exhibited high activity against filamentous fungi, *Trichophyton rubrum, Microsporum* gypseum, and *Penicillium marneffei* with IC50 values of 0.2, 0.2 and 0.4 mg/ml, respectively. However, it showed moderate activity against yeasts: *Candida albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae* (MIC 0.1-1 mg/ml) and low activity against bacteria (MIC 5-10 mg/ml). Results presented here on the antimicrobial activities of the rhizome and leaf extracts completely matches with those reported by Phongpaichit et al. (2005). Nevertheless, there are slight differences in the MIC and zone of inhibition values. Also, there are reports available suggesting that antimicrobial properties of the plant parts or whole plants vary with the type of solvent used to prepare the extracts from respective plant parts. Extracts of *A. calamus* rhizomes obtained with dichloromethane and ethanol (Mungkornasawakul 2000; Thirach et al. 2003) has been reported to exhibit substantial antifungal activity. The ethanol extract of *A. calamus* inhibited clinical isolates of *C. albicans*. Therefore from several previous studies it has become clear that the differences in the effectiveness (MIC value) could be due to the solvents used for extraction of active ingredients from the concerned plant parts or climatic and geographical differences. The sensitivity of the microorganisms to the rhizome and leaf extract could be due to morphological as well as

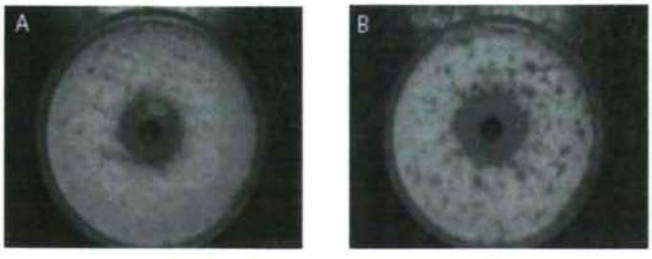


Figure 1. Well diffusion assay of rhizome [A] and leaves [B] extracts exhibiting antifungal activity against *Aspergillus flavis*.

difference in the cell wall constitution of the microorganisms tested. Opposite to the previous studies, ethyl acetate in the present study has been found to be a best solvent for extraction of the active ingredient (α- and β-asarone) from rhizome and leaves among other solvents. Though, solvents such as methanol, ethanol and hexane used in most of the previous studies were also found to be suitable for extraction of active ingredients. It is well established that the α- and β-asarones found in leaf, roots and rhizome tissues are responsible for almost all of the antimicrobial activities of the *A. calamus* (MacGaw et al. 2002). Leet et al. (2007) have recently investigated fungicidal property of *A. gramineus* rhizome (methanol extract) against phytopathogenic fungi apart from human pathogenic microorganisms. They attributed these antifungal activities to the α-asarone and asaronaldehyde present in *A. gramineus*. In our study, we confirmed the presence of α- and β-asarones in *A. calamus* rhizome and leaf extracts by thin layer chromatography (TLC). Antimicrobial activities of *A. calamus* reported here thus could be attributed to the α- and β-asarones found in the rhizome and leaf extracts. To substantiate these results, we investigated the antimicrobial activity of authentic α- and β-asarones. Both α- and β-asarones demonstrated strong antifungal and antiyeast activities but not the antibacterial activity. These results clearly suggested that the antimicrobial activity of the rhizome and leaf extracts was due to the presence of α- and β-asarones. Nevertheless, both α- and β-asarones have comparatively higher microbial growth inhibition potential than the rhizome and leaf extracts because of their purity. Finally, when antimicrobial activities of the extracts of the respective plant parts and pure α- and β-asarones were compared to those of standard streptomycin and amphotericin B it revealed that pure α- and β-asarones possess significant antimicrobial activity while rhizome and leaf extracts possess only considerable antimicrobial activity. However, purification of the crude extract will enhance the antimicrobial activity of the concerned plant parts. Currently biosynthesis of α- and β-asarones along with their bioactive potential is being studied in our laboratory.

Acknowledgement

Authors are grateful to Mr. G. Viswanathan, Chancellor, VIT University, Vellore for providing necessary support and facilities.

References

Bertea CM, Azzolin CMM, Bossi S, Doglia G, Maffei ME (2005) Identification of an EcoR1 restriction site for rapid and precise determination of β-asarone-free *Acorus calamus* crtotypes. Phytochemistry 66:507-514

De M, De AK, Banerjee AB(1999) Antimicrobial screening of some Indian spices. Phototherapy Res 13:616-618.

Grosvenor PW, Suprino A, Gray DO (1995) Medicinal plants from Riau Province, Sumatra,Indonesia. Part 2: antibacterial and antifungal activity. J Ethnopharmacol 45:97-111.

Koul O, Saxena BP, Tikku K (1977a) Mode of action of *Acorus calamus* L.oil vapours on adult male sterility in red cotton bugs. Experientia 33:29-31.

Koul O, Saxena BP, Tikku K (1977b) Follicular regression in Trogoderma granarium due to sterilizing vapours of *Acorus calamus* L. oil. Cur Sci 46:724-725.

Lander V, Schreier P (1990) Acorenone and c-asarone: indicators of the origin of Calamus oils (*Acorus calamus* L.). Flavor Fragrance J 5:75-79.

Lee HS (2005) Pesticidal constituents derived from Piperaceae fruits. Agric Chem Biotechnol 48:65-74.

Lee HS (2007) Fungicidal property of active component derived from Acorus gramineus rhizome against phytopathogenic fungi. Bioresource Technol 98:1324-1328.

Lee JY, LeeJY, Yun BS, Hwang BK (2004) Antifungal activity of β-asarone from rhizomes of *Aocrus gramineus*. J Agric Food Chem 52:776-780.

Lorian V (1996) Antibiotics in Laboratory Medicine, fourth ed. Williams and Wilkins, Baltimore.

Mathur AC, Saxena BP (1975) Induction of sterility in male house flies by vapours of *Acorus calamus* L. oil. Naturwissenschaften 62:576.

McGaw LJ, Jager AK, Van Staden J (2002) Isolation of β-asarone, an antibacterial and anthelmintic compound, from *Acorus calamus* in South Africa. South African J Bot 68:31-35.

Mehrotra S, Mishra KP, Maurya R, Srimal RC, Yadav VS, Pandey R, Singh VK (2003) Anticellular and Immunosuppressive Properties of Ethanolic extract of *Acorus calamus* rhizome. International Immunopharmacol 3:53-61.

Mungkornasawakul, P (2000) Fungicide from Acorus calamus Linn., Eugenia caryophyllus Bullock et Harrison and Mammea siamensis Kosterm. And their Residues after Application. M. Sc. Thesis. Chiang Mai University. Chiang Mai.

Namba T (1993) The encyclopedia of Wakan-Yuku (tranditional Sino Japanese Medicines, with color pictures). Hoikusha. Osaka Japan 1:606.

Nawamaki K, Kuroyanagi M (1996) Sesquiterpenoids from *Acorus calamus* as germination inhibitors. Phytochemistry 43:1175-1182.

Oprean R, Tamas M, Roman L (1998) Comparison of GC-MS and TLC techniques for asarone isomers determination1. J Pharm Biomed Anal 18:227-234.

Phongpaichit S, Pujenjob N, Rukachaisirikul V, Ongsakul M (2005) Antimicrobial activities of the crude methanol extract of *Acorus calamus* Linn. M J Sci Technol 27:517-523.

Priya K, Deepak Ganjewala (2007) Antibacterial activities and Phytochemical Analysis of different Plant Parts of Nyctanthes arbor-tristis (Linn.). Res J Phytochem 1:61-67.

Raina VK, Srivastava SK, Syamasunder KV (2003) Essential oil composition of Acorus calamus L. from the lower region of the Himalayas. Flavour Fragrance J 18:18-20.

Rani AS, Satyakala M, Devi VS, Murty US (2003) Evaluation of antibacterial activity from rhizome extracts of *Acorus calamus* Linn. J Sci Indust Res 62:623

Rani AS, Subhadra VV, Reddy VD (2000) In vitro propagation of Acorus calamus Linn.--a medicinal plant. Indian J Exp Biol 38:730-732

Saxena BP, Koul O, Tikku K, Atal CK (1977) A new insect chemosterilant isolated from *Acorus calamus* (L.). Nature 270:512-513.

Schmidt GS, Brochers D (1981) Studies of the sterilizing effect of Indian *Acorus calamus* in ants. Mitt Dtsch Ges Allg Angew Entomol 3:201-213.

Thirach S, Tragoolpua K, Punjaisee S, Khamwan C, Jatisatiennr C, Kunyanone N (2003) Antifungal activity of some medicinal plant extracts against *Candida albicans* and *Cryptococcus neoforman*. Acta Hort (ISHS) 597:217-221.

Venskutonis PR, Dagilyte A (2003) Composition of essential oil of sweet flag (*Acorus calamus* L.) leaves at different growing phases. JEOR 15:313-318.

Wang HZ, Cheng YG, Fan CS (1998) Review of studies on chemical constituents and pharmacology of genus Acorus. Acta Bot Yunnanica 5:96-100.

Volume 53(1):51-54, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Rapid PCR based identification of two medically important dermatophyte fungi, *Microsporum canis* and *Trichophyton tonsurans*

Gabriela Malinovschi[1]*, Sándor Kocsubé[2], László Galgóczy[2], Ferenc Somogyvári[3] and Csaba Vágvölgyi[2]

[1]Department of Dermatology, University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj Napoca, Romania, [2]Department of Microbiology, University of Szeged, Szeged, Hungary, [3]Department of Medical Microbiology and Immunology, University of Szeged, Hungary

ABSTRACT PCR-based species specific protocols have been worked out for two important pathogens *M. canis* and *T. tonsurans* responsible for dermatophytosis with various clinical appearances. Reactions were designed to use a common reverse primer and 2 specific forward primers and were optimised to be efficient under the same PCR conditions allowing the detection of these two fungi from one reaction volume. The specific PCR reactions were evaluated both on collection strains of other dermatophytes and on clinical samples. In connection with these methods, different DNA extraction techniques were also tested to assist for an efficient PCR based detection.

Acta Biol Szeged 53(1):51-54 (2009)

KEY WORDS

dermatophytes
DNA extraction
ITS
polymerase chain reaction

The dermatophytes are morphologically and physiologically related moulds widely distributed throughout the world. The most frequently encountered species belong to the genera *Epidermophyton*, *Microsporum*, and *Trichophyton*: as keratin utilizing microorganisms they frequently infect the hair, skin and nails. An atypical manifestation with more severe and more extensive lesions can evolve in immunocompromised patients. Direct contact or exposure to infected desquamated cells can promote transmission (Santos et al. 2006). A number of exoenzymes, such as elastase, keratinases and proteinases, which can facilitate their invasion into keratinized tissues, are the important virulence factors of these fungi (Weitzman and Summerbell 1995). Though the daily routine in the drug selection against these fungi rarely relies on the exact recognition of the microroganism, however, future trends for more efficient treatments and the need to know more about their epidemiology underline the importance of the molecular species identification. Nowadays, this is a relatively neglected field for dermatophytes. While there is a plethora of various molecular methods for most of the groups of human pathogenic fungi, in case of dermatophytes, only sporadic attempts appear in the literature (e.g.: Jackson et al. 1999, 2000; Liu et al. 2000; Arabatzis et al. 2007).

In the absence of a functional dermatophyte-specific polymerase chain reaction (PCR), current diagnosis of dermatophytoses relies on microscopy and culture. However, the combination of these techniques is particularly time-consuming and low in sensitivity.

The aim of the present study was to develop a molecular method allowing the species specific identification of *Microsporum canis* and *Trichophyton tonsurans*. In connection with this, different DNA extraction methods were tested as well as specific polymerase chain reaction (PCR) based methods were worked out and evaluated for these species.

Materials and Methods

Strains

The following culture collection strains were used in this study: *Microsporum canis* (American Type Culture Collection, USA; ATCC 36299), *Microsporum gypseum* (ATCC 24102), *Trichophyton mentagrophytes* (ATCC 9533), *Trichophyton rubrum* (ATCC 28188) and *Trichophyton tonsurans* (ATCC 28942). Seventeen dermatophytes isolated from randomly selected clinical samples (nail and skin) were also used in the evaluation of the method. Clinical samples were collected at the Department of Dermatology, University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj Napoca (Kolozsvár), Romania. All these isolates were maintained on potato dextrose agar (PDA, Sigma, 0.4% potato starch, 2% glucose, 1.5% agar) slants at 4°C.

DNA extraction

For DNA extraction, dermatophytes were grown in potato dextrose liquid medium (PDB) under continuous shaking

Accepted July 2, 2009
*Corresponding author. E-mail: gmalinovschi@yahoo.com

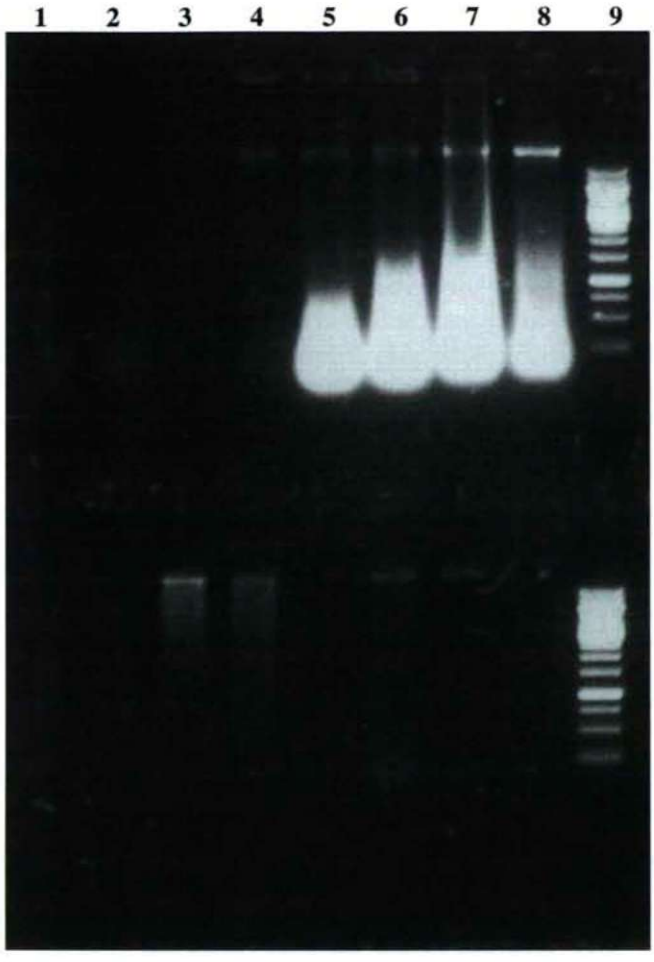


Figure 1. Comparison of DNA fragment size distributions and relative DNA yields obtained with the four DNA extraction procedures described in the Materials and Methods section. Five-microliter portions from these extractions were electrophoresed on a 0.8% (wt/vol) agarose/TAE gel. Upper and lower row, lane 9: GeneRuler 1 kb DNA Ladders (Fermentas). Lanes 1-4 and 5-8 (in both rows) contains DNA from *T. mentagrophytes*, *T. tonsurans*, *M. canis* and *M. gypseum*, respectively. Upper row, lanes 1-4 and 5-8: E.Z.N.A.® Yeast DNA Kit and MasterPure Yeast DNA Purification Kit, respectively. Lower row, lanes 1-4 and 5-8: ZR Fungal/Bacterial DNA Kit and Qiagen DNeasy Plant Mini Kit, respectively.

(200 rpm) at 30°C for 7 days. Four commercially available DNA extraction kits were tested and used in the total DNA extraction experiments: ZR Fungal/Bacterial DNA Kit (Zymo Research, Orange, CA, USA), MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), Qiagen DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) and E.Z.N.A.® Yeast DNA Kit (Omega Biotek, Norcross, GA, USA). All these kits were used according to the instructions of the manufacturers. DNA samples were stored at -20°C.

PCR reactions and electrophoresis

Species specific PCR reactions were performed as follows: each 20 µl of reaction mixture contained 2 µl of 10 x PCR buffer (Double-Taq, ZenonBio), 4 µl of dNTP solution (200 µM each of dATP, dCTP, dGTP and dTTP), 3 µl of 25 mM $MgCl_2$ (ZenonBio), 4 - 4 µl (0.2 - 0.2 µM) of primers, 0,2 µl (0.5 U) of Double-Taq DNA polymerase (ZenonBio), 1 µl of genomic DNA extract (50 ng/µl) and 1.8 µl distilled water. Control reactions, without genomic DNA extract, were also run.

Amplifications were performed with a PTC-0148 Mini48 DNA thermocycler (BioRad, USA). The first cycle involved a denaturation step at 94°C for 2 min. This was followed by 35 amplification cycles, involving a denaturation step at 94°C for 10 sec, an annealing step at 67°C for 20 sec and a chain extension step at 72°C for 1 min.

Identification of clinical samples has been performed by the amplification of the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) followed by the sequencing of the amplification products (Luo and Mitchell 2002). The PCR primers used were ITS1 and ITS4 which amplify the variable ITS1 and ITS2 sequences surrounding the 5.8S-coding sequence and situated between the Small SubUnit-coding sequence and the Large SubUnit-coding sequence of the ribosomal operon (White et al. 1990). The conditions of the PCR were as described by Saiki et al. (1988). The reaction mixture (20 µl) contained 2 µl of 10 x PCR buffer (Double-Taq, ZenonBio), 4 µl of dNTP solution (200 µM each of dATP, dCTP, dGTP and dTTP), 3 µl of 25 mM $MgCl_2$ (ZenonBio), 4 - 4 µl (0.2 - 0.2 µM) of ITS1 and ITS4 primers, 0.2 µl (0.5 U) of Double-Taq DNA polymerase (ZenonBio), 1 µl of template DNA (50 ng/µl), 1 µl BSA (Fermentas) and 0.8 µl sterile distilled water.

Ten µl of each amplification product was separated by electrophoresis on 1% agarose/TAE (4.84 g Tris base, 1.14 glacial acetic acid, 2 ml 0.5 Na EDTA pH=8) gels and visualized by UV fluorescence (UVP, BioDoc-It™) after ethidium bromide (0.5 mg/ml) staining, using GeneRuler 100 bp Plus DNS marker (Fermentas) as size standard.

Results and Discussion

Nucleic acid extraction from dermatophytes

Protocols for extraction of DNA of fungal cells either are very time-consuming or show poor yield of DNA compared to methods of extraction of DNA, e.g. of human cells. Other protocols require additional lysis steps like sonification or mechanical disruption or harmful chemicals such as phenol-chloroform or guanidine thiocyanate. From these reasons, commercial nucleic acid extraction and purification kits are more and more popular. Nucleic acid samples with proper quality are important prerequisite for any further DNA associated identification method. This especially important for the dermatophytes providing minuscule samples from clinical sources and growing very slowly under laboratory conditions.

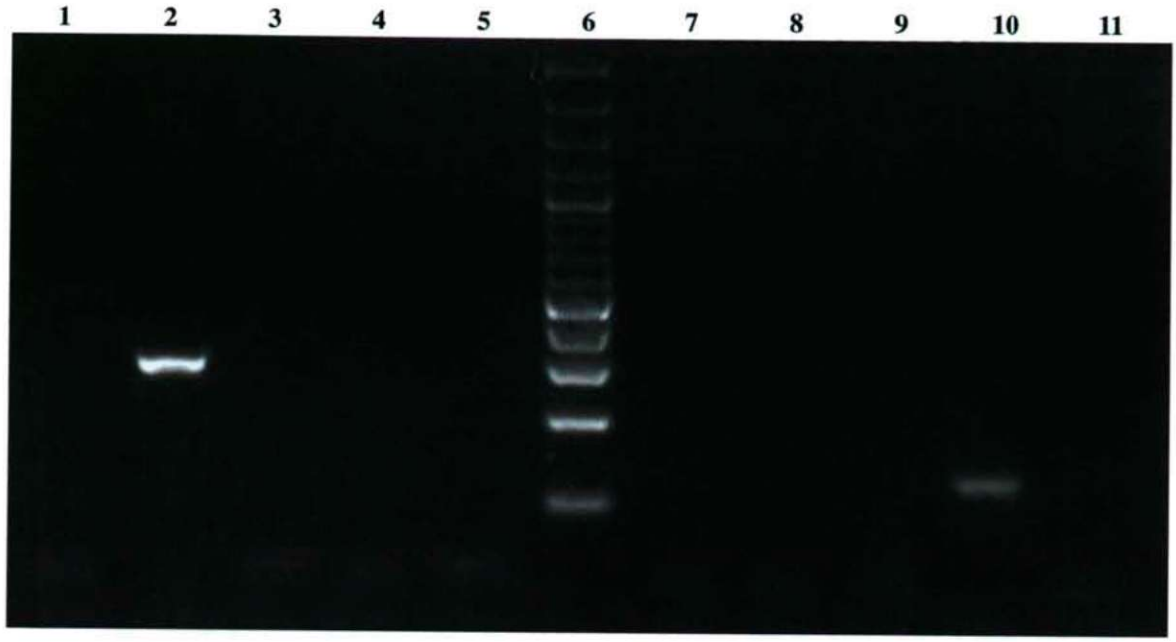


Figure 2. Amplification products of the PCR reactions with species specific primers for *T. tonsurans* (TTF/CR; lanes 1-5) and *M. canis* (MCF/CR; lanes 7-11), electrophoresed on 3% agarose gel. Lanes 1-5 and lanes 7-11: *T. mentagrophytes, T. tonsurans, T. rubrum, M. canis* and *M. gypseum*, respectively. Lane 6: GeneRuler 100 bp Plus DNA marker (Fermentas).

Commercial kits tested in this study utilise little bit different approaches for nucleic acid isolation. The E.Z.N.A.® Yeast DNA Kit combines the reversible nucleic acid-binding properties of HiBind® matrix with spin column technology. The MasterPure™ Yeast DNA Purification Kit's protocol involves nonenzymatic cell lysis at 65°C, followed by removal of protein by precipitation, and nucleic acid precipitation and resuspension. No lyticase, proteolytic enzymes, or bead-beating are used in the procedure. The ZR Fungal/Bacterial DNA Kit™ disrupts cells by bead beating; nucleic acids are further purified by spin column technology (ZR BashingBead™ Lysis/Filtration Tube) without using organic denaturants or proteinases. The DNeasy Plant Kits (Qiagen) provides silica-based DNA purification in spin columns: no organic extraction or ethanol precipitation is necessary for the procedure.

Among the four kits tested, MasterPure Yeast DNA Purification Kit (Epicentre) proved to be a very efficient tool for isolation DNA for dermatophytes. When tested on 4 different species (*T. mentagrophytes*, *T. tonsurans*, *M. canis* and *M. gypseum*) this protocol provided the highest yield and reproducibility (Fig. 1).

Table 1. Primers used in PCR reactions.

Primer	Sequence (5'-3')
CR (*common reverse*)	5' – TCGCTGCGTTCTTCATCGATG – 3'
MCF	5' – CGCTCGCCGGAGGATTACTC – 3'
TTF	5' – AGGATAGGGCCAAACGTCCGT – 3'

Species specific identification of *M. canis* and *T. tonsurans*

Traditionally, identifications of dermatophytes were performed using conventional methods of mycological identification which are based on the analysis of characteristic macroscopic and microscopic features of these fungi. However, such identification is difficult to perform due to the polymorphic character of these traits, additionally increased by variations in temperature, media composition, and other parameters of cultivation. Likewise, in some instances, the dermatophytes fail to produce any obvious reproductive structure in culture (sterile mycelia) which makes it impossible for ultimate diagnosis.

Molecular methodologies provide powerful new tools for the identification of various pathogenic fungi (White et al.1990; Luo and Mitchell 2002; Borman et al. 2008; Nyilasi et al. 2008). Among these, PCR-based methods are particularly promising in connection with the identification of dermatophytes because of their simplicity, specificity, and sensitivity (Jackson et al. 1999; Mochizuki et al. 2003; Ohst et al. 2004; Arabatzis et al. 2007).

Whereas in the genus *Epidermophyton*, *E. floccosum* is the only species which is pathogenic, the genera *Microsporum* and *Trichophyton* are made up of multiple anthropophilic and zoophilic species. Furthermore, besides the several species

of *Trichophyton*, several different variants are also identified within the species *T. mentagrophytes* (Gräser et al. 1999; Liu et al. 2000).

In the present study, PCR-based species specific protocols have been worked out for two important pathogens, *M. canis* and *T. tonsurans*. The sizes of amplicons from these 2 specific reactions are substantially different (128 bp, 285 bp; Fig. 2). Reactions were designed to use a common reverse primer (CR) and 2 specific forward primers (MCF, TTF; Table 1) and were optimised to be efficient under the same PCR conditions allowing the detection of these two fungi in one reaction.

When this set of primers was tested for other frequently encountered dermatophytes no false amplifications were detected (Fig. 2). They were also evaluated on clinical samples where the identity of the pathogens was later proved by sequencing the ITS gene regions. These 17 samples contained the following fungi: *T. tonsurans*, *T. rubrum* (9), *T. mentagrophytes* (3), *M. canis* (2), *M. gypseum*, and one unidentified fungus. Both *T. tonsurans* and *M. canis* were reproducible detected from these samples without the cross-reaction with other fungi (results not shown).

In our study specific PCR reactions were developed for two dermatophytes, *M. canis* and *T. tonsurans*. Further investigations with the aim to establish similar identification reactions for other frequently encountered dermatophytes are in progress now.

Acknowledgements

This investigation was financially supported by the OTKA/DFG grant NN 75255.

References

Arabatzis M, Bruijnesteijn van Coppenraet LES, Kuijper EJ, de Hoog GS, Lavrijsen APM, Templeton K, van der Raaij-Helmer EMH, Velegraki A, Gräser Y, Summerbell RC (2007) Diagnosis of common dermatophyte infections by a novel multiplex real-time polymerase chain reaction detection/identification scheme. Brit J Dermatol 157:681-689.

Borman AM, Linton CJ, Miles S-J, Johnson EM (2008) Molecular identification of pathogenic fungi. J Antimicrob Chemoth 61:S1, i7–i12, doi:10.1093/jac/dkm425.

Gräser Y, Kuijpers AFA, Presber W, De Hoog GS (1999) Molecular taxonomy of *Trichophyton mentagrophytes* and *T. tonsurans*. Med Mycol 37:315-330.

Jackson CJ, Barton RC, Evans EGV (1999) Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal DNA intergenic spacer regions. J Clin Microbiol 37:931-936.

Jackson CJ, Barton RC, Kelly SL, Evans EGV (2000) Strain identification of *Trichophyton rubrum* by specific amplification of subrepeat elements in the ribosomal DNA nontranscribed spacer. J Clin Microbiol 38:4527-4534.

Liu D, Coloe S, Baird R, Pedersen J (2000) Application of PCR to the identification of dermatophyte fungi. J Med Microbiol 49:493-497.

Luo G, Mitchell TG (2002) Rapid identification of pathogenic fungi directly from cultures by using multiplex PCR. J Clin Microbiol 40:2860-2865.

Mochizuki T, Ishizaki H, Barton RC, Moore MK, Jackson CJ, Kelly SL, Evans EG (2003) Restriction fragment length polymorphism analysis of ribosomal DNA intergenic regions is useful for differentiating strains of *Trichophyton mentagrophytes*. J Clin Microbiol 41:4583-4588.

Nyilasi I, Papp T, Csernetics Á, Krizsán K, Nagy E, Vágvölgyi Cs (2008) High-affinity iron permease (FTR1) gene sequences-based molecular identification of clinically important Zygomycetes. Clin Microbiol Infect 14:393-397.

Ohst T, de Hoog S, Presber W, Stavrakieva V, Gräser Y (2004) Origins of microsatellite diversity in the *Trichophyton rubrum–T. violaceum* clade (dermatophytes). J Clin Microbiol 42:4444-4448.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science 239:487-491.

Santos DA, Barros MES, Hamdan JS (2006) Establishing a method of inoculum preparation for susceptibility testing of *Trichophyton rubrum* and *Trichophyton mentagrophytes*. J Clin Microbiol 44:98-101.

Weitzman I, Summerbell RC (1995) The dermatophytes. Clin Microbiol Rev 8:240-59.

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis MA, Gelfand DH, Sninsky JJ, White TJ, ed., PCR Protocols: A Guide to Methods and Applications. New York: Academic Press Inc; 1990. pp. 315-322.

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

DISSERTATION SUMMARIES

The role of the orange carotenoid protein (OCP) and PsbU subunit in photoinhibition of Photosystem II in cyanobacteria

Leyla Abasova

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Excess light is harmful for photosynthetic organisms. In *Synechocystis* 6803, high intensities of blue-green light induce Photosystem II fluorescence quenching which is specifically associated with a photoprotective phycobilisome related energy-dissipation mechanism. A soluble orange carotenoid protein (OCP), of previously unknown function, plays an essential role in this process (Wilson et al. 2006). In the absence of the OCP, the non-photochemical quenching (NPQ) induced by strong blue-green light in *Synechocystis* PCC 6803 cells is completely inhibited and the cells are more sensitive to high light intensities. In iron-starved *Synechocystis* 6803 cells a larger OCP-phycobilisome-related NPQ is observed in association with a higher concentration of OCP (Wilson et al. 2007). Highly conserved homologues of the OCP are found in almost all genomes of all cyanobacteria for which genomic data is available with the exception of *Synechoccocus elongatus*, *Thermosynechoccocus elongatus* and the *Prochlorococci*.

The existence of a blue-green light induced NPQ mechanism was tested in iron-containing and iron-depleted cells of *A. maxima* strain, containing OCP genes, and *T. elongatus* and *S. elongatus* PCC 7942 strains lacking the entire OCP gene. The kinetics of fluorescence changes was monitored by a modulated PAM (pulse-amplitude-modulated) fluorometer. Exposure of low blue light–adapted cells to high blue-green light intensities induced a quenching of the Fm' in OCP containing *A. maxima* strain. In contrast, illumination of OCP lacking *S. elongatus* and *T. elongatus* by strong blue-green light did not induce any decrease of Fm' indicating that in the absence of whole OCP gene this kind of photoprotective quenching cannot be induced. We tested possible relationship between the iron starvation and the blue-green light–induced NPQ.

Results obtained from fluorescence traces of 10 days iron-starved cells of *A. maxima* demonstrates increase of fluorescence quenching in compare with iron containing cells. This increase, like in a case of *Synechocystis* cells, can be related to larger accumulation of OCP under iron starvation. In contrast, exposure of prolonged iron starved *T. elongatus* and *S. elongatus* PCC 7942 to high intensities of light didn't induce any NPQ. Iron starvation induces the synthesis of the "chlorophyll-binding-iron-stressed induced protein", IsiA in all strains. The presence of IsiA causes a blue-shift in the room temperature Chl *a* absorbance peak (680-673 nm). Under iron stress conditions both chlorophyll and phycobilisome level decreased in all cells. According to the absorbance spectra iron starved *T. elongatus* and *S. elongatus* PCC 7942 cells show different reorganisation of the pigments compared to OCP containing *A.maxima and Synechocystis* cells: phycobiliprotein content decreased faster than chlorophyll content. Increase at 685 nm in fluorescence spectra generated at 77K by 430 nm in iron-starved *A. maxima* cells was attributed to the accumulation of uncoupled phycobilisomes with high fluorescence emission as previously shown in iron-starved *Synechocystis* cells. In contrast, in *T elongatus* and *S. elongatus* PCC 7942 cells the 685 nm emission (and 660 nm emission) decreased with the time instead of increase like in *A.maxima* cells. Thus, in *T elongatus* and *S. elongatus* PCC 7942 cells phycobiliprotein content decreased faster than chlorophyll content. This is probably essential for the longer survival of the cells in the absence of the photoprotective blue-green light induced NPQ mechanism (Boulay et al. 2008).

Photosystem II contains trans-membrane protein subunits and extrinsic proteins associated with the lumenal side. While great progress has been made in the understanding of the function of the water splitting apparatus, the role of the smaller subunits is still not well defined. Studies of deletion mutants demonstrated the importance of these proteins in stabilising the Mn cluster. PsbU is one of the extrinsic proteins which form the oxygen-evolving complex of PS II in cyanobacteria (Burnap et al. 1992). In this study we investigated the function of PsbU using a deletion mutant of the *psbU* gene in *S. elongatus* PCC 7942 (Balint et al. 2006). Flash-induced chlorophyll fluorescence measurements were used to monitor forward electron transfer at the acceptor side of PSII, as well as charge recombination processes of the reduced acceptors with oxidized donor side components. While forward electron transfer was similar between the wild type and the PsbU strains, a marked difference in the rate of back electron transfer in the presence of electron transfer inhibitor DCMU was observed in the mutant.

The overall fluorescence relaxation kinetics in PsbU mutant in the presence of DCMU resulted in a slow phase of 1.2 s and 82% amplitude whereas in mutant the corresponding values were 4.8 s, 64%. Moreover, thermoluminescence measurements have demonstrated that in the presence and absence of DCMU, the TL intensity in mutant cells was significantly increased, and this was accompanied by the shift of the peak position to higher temperatures for both the Q- and B-bands. Also the high light induced loss of oxygen-evolving activity in the presence and absence of an inhibitor of protein synthesis lyncomicin was monitored in the PsbU and wild type cells. When the high light illumination was performed in the presence of the protein synthesis inhibitor lincomycin the activity loss was significantly higher in mutant cells than in wild type. On the basis of these results, we conclude that PsbU is crutial for the stable architecture of the PSII.

Balint I, Bhattacharya J, Perelman A, Schatz D, Moskovitz Y, Keren N, Schwarz R (2006) Inactivation of the extrinsic subunit of photosystem II, PsbU, in *Synechococcus* PCC 7942 results in elevated resistance to oxidative stress. FEBS Lett 580(8):2117-2122.

Boulay C, Abasova L, Six C, Vass I, Kirilovsky D (2008) Occurrence and function of the orange carotenoid protein in photoprotective mechanisms in various cyanobateria. Biochim Biophys Acta 1777(10):1344-1354.

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Wilson A, Ajlani G, Verbavatz JM, Vass I, Kerfeld CA, Kirilovsky D (2006) A soluble carotenoid protein involved in phycobilisome-related energy dissipation in cyanobacteria. Plant Cell 18(4):992-1007.
Wilson A, Boulay C, Wilde A, Kerfeld CA, Kirilovsky D (2007) Light-induced energy dissipation in iron-starved cyanobacteria:roles of OCP and IsiA proteins Plant Cell 19(2):656-672.

Supervisor: Imre Vass
E-mail: labasova@brc.hu

The expression of ABCC4 and ABCG2 xenobiotic transporters during keratinocyte proliferation/differentiation and in psoriasis

Attila Bebes

Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary

Xenobiotic transporters are members of the ATP binding cassette (ABC) superfamily of proteins, responsible for the energy dependent transport of a broad range of chemically and structurally different compounds thus provide chemoresistance for various tumors. However, they also play a very important role in maintaining the chemical barrier function of organs such as brain, liver and gut (see for review Leslie et al. 2004). The human epidermis is one of the largest physical and biochemical barrier of the body. There have been only a few studies conducted regarding xenobiotic transporter expression in normal human keratinocytes and in human skin (Baron et al. 2001; Kielar et al. 2003).

We aimed to study the expression of eight xenobiotic transporters: ABCB1, ABCC1-6 and ABCG2 in in vitro models of keratinocyte differentiation. Terminal differentiation of normal human keratinocytes was promoted by increasing Ca^{2+} concentration. Validation of the differentiation model was achieved by the detection of proliferation markers Ki67 and integrin alpha 5 and differentiation markers keratin 1 and involucrin. The chemical-free synchronization of the immortalized keratinocyte cell line, HaCaT was used as another model (Pivarcsi et al. 2001), in which contact inhibiton and serum starving forces the cells into a highly differentiated quiescent state. Releasing HaCaT keratinocytes from cell quiescence by passaging and serum re-addition initiate redifferentiation and the cells start to proliferate synchronously.

Among the transporter genes tested ABCC4 and ABCG2 showed a proliferation associated expression in both *in vitro* models. ABCC4 and ABCG2 were highly expressed in undifferentiated, proliferating keratinocytes and their mRNA levels decreased in parallel with differentiation. ABCC4 and ABCG2 transporter protein levels also showed a decrease in differentiating keratinocytes, as revealed by Western blot and immunocytochemistry. Similarly, induction of ABCC4 and ABCG2 mRNAs and proteins were observed in synchronized HaCaT keratinocytes after release from cell quiescence, which further supported that ABCC4 and ABCG2 transporters have a possible function in proliferating keratinocytes.

ABCC4 protein was overexpressed in the basal layers of psoriatic lesional epidermis, supporting our *in vitro* results, while in keratinocytes of normal and non-involved skin it was expressed at very low levels. ABCC4 transporter may contribute to the pathogenesis of psoriasis since antiapoptotic/proliferation related cyclic nucleotides and important inflammatory mediators are ABCC4 substrates. ABCG2 transporter was expressed in normal and psoriatic non-involved epidermis and its expression was restricted to basal layer keratinocytes. Increased levels of ABCG2 protein was detected in psoriatic lesions, however its highest level of expression was observed in keratinocytes in the abnormally differentiating granular layer. It is known that human epidermis is a constitutively hypoxic tissue, which is more pronounced in psoriatic lesions. Hypoxia induces the generation of harmful porphyrins that are substrates of ABCG2, thus ABCG2 may protect keratinocytes from the accumulation of these compounds. The upregulation of ABCC4 and ABCG2 xenobiotic transporters in psoriatic lesions could significantly modulate drug distribution and effectiveness in the skin.

Baron JM, Höller D, Schiffer R, Frankenberg S, Neis M, Merk HF, Jugert FK (2001) Expression of multiple cytochrome P450 enzymes and multidrug resistance-associated transport proteins in human skin keratinocytes. J Invest Dermatol 116(4):541-548
Kielar D, Kaminski WE, Liebisch G, Piehler A, Wenzel JJ, Möhle C, Heimerl S, Langmann T, Friedrich SO, Böttcher A, Barlage S, Drobnik W, Schmitz G (2003) Adenosine triphosphate binding cassette (ABC) transporters are expressed and regulated during terminal keratinocyte differentiation: a potential role for ABCA7 in epidermal lipid reorganization. J Invest Dermatol 121(3)465-474
Leslie EM, Deeley RG, Cole SPC (2005) Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2 and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol 204:216-237

Supervisor: Márta Széll, István Raskó
E-mail: bebes.attila@gmail.com

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Characterization of the novel opioid and nociceptin peptides

Engin Bojnik

Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

Opioid system is consists of μ, δ, κ and nociceptin (NOP) receptors and their respective endogenous neuropeptide ligands. In this study we have characterized novel non-mammalian opioid peptides Ile-enkephalin and Phe-enkephalin, and artificial NOP receptor partial agonist hexapeptide Ac-RYYRIR-ol.

Leu- and Met-enkephalin were the first endogenous opioid peptides identified in different mammalian species including the human. Comparative biochemical and bioinformatic evidence indicates that enkephalins are not limited to mammals. Lower vertebrate enkephalins were investigated with in vitro biochemical experiments using rat brain membrane preparations and turned out to be moderate affinity opioids with a definite preference for the δ-opioid receptor sites. Phe-enkephalin from the lungfish displayed low affinities toward the μ- and δ-opioid receptor, while exhibited moderate affinity toward the κ-opioid receptor. In [^{35}S]GTPγS binding studies, Met-enkephalin produced the highest stimulation followed by Leu-enkephalin, Ile-enkephalin from the clawed frog and Phe-enkephalin, was the least efficacious among these endogenous peptides (Bojnik et al. 2009a).

Some N/OFQ sequence unrelated hexapeptides can effectively bind to the NOP receptor and they were used as template for structure activity studies that lead to discovery of the new NOP selective ligands. The pharmacological profile of the novel hexapeptide Ac-RYYRIR-ol was investigated using various in vitro assays including receptor binding and G protein activation in rat brain membranes, mouse vas deferens, rat vas deferens, guinea pig ileum, mouse colon and calcium mobilization. In rat brain membranes Ac-RYYRIR-ol displaced [^{3}H]Ac-RYYRIK-ol (Bojnik et al. 2009b) with high affinity and stimulated [^{35}S]GTPγS binding with high potency. The stimulatory effect of Ac-RYYRIR-ol was antagonized by the selective NOP receptor antagonist UFP-101. In antagonist type experiments Ac-RYYRIR-ol inhibited the stimulatory effects induced by N/OFQ. In the electrically stimulated mouse vas deferens Ac-RYYRIR-ol displayed negligible agonist activity while antagonizing in a competitive manner the inhibitory effects of N/OFQ. In the mouse colon Ac-RYYRIR-ol produced concentration dependent contractile effects with similar potency and maximal effects as N/OFQ. Finally, in the Ca^{2+} mobilization assays Ac-RYYRIR-ol displayed lower potency and maximal effects compared with N/OFQ assays.

In conclusion, two novel, non-mammalian enkephalins were described and compared with those of the well-known Leu- and Met-enkephalin. Among the four structures tested, the 'mammalian type' Met-enkephalin exhibited the highest affinities in receptor binding assays and produced the most efficacious G-protein stimulation in brain membranes, while the newly identified 'lower vertebrate type' Ile- and Phe-enkephalins were found to be less effective. On the other hand, novel NOP receptor selective hexapeptide Ac-RYYRIR-ol has been shown to have fine selectivity, high potency, furthermore agonist and antagonist effects toward the NOP receptors were measured in various assays. This is likely due to its partial agonist pharmacological activity.

Bojnik E, Magyar A, Tóth G, Bajusz S, Borsodi A, Benyhe S (2009a) Binding studies of novel, non-mammalian enkephalins, structures predicted from frog and lungfish brain cDNA sequences. Neuroscience 158:867-874.

Bojnik E, Farkas J, Magyar A, Tömböly C, Güclü U, Gündüz O, Borsodi A, Corbani M, Benyhe S (2009b) Selective and high affinity labeling of neuronal and recombinant nociceptin receptors with the hexapeptide radioprobe [3H]Ac-RYYRIK-ol. Neurochemistry International (in press).

Supervisor: Anna Borsodi
E-mail: engin@brc.hu

Genetic modification of carotene producing Zygomycetes

Árpád Csernetics

Department of Microbiology, University of Szeged, Szeged, Hungary

Carotenoids are terpenoid-type chemical compounds. These yellow to orange-red natural pigments are used in the food, pharmaceutical and cosmetic industry and as feed colour additives. Recently, they are attracting an increasing attention, due to their beneficial effects on health. In Zygomycetes fungi, β-carotene is the predominant carotenoid. Traditionally three species: *Blakeslea trispora*, *Phycomyces blakesleeanus* and *Mucor circinelloides* have been involved in the study of the carotene biosynthesis.

Mucor circinelloides has several characteristics advantageous for molecular genetic studies. For example, well functioning methods are available for the genetic transformation of this fungus based on autonomously replicating plasmids (Papp et al. 2008). However, integrative transformation methods are not well established and the fate of the transforming DNA has not yet been analyzed.

The aims of our work were (1) to investigate and compare the effect of overexpression of different isoprene biosynthesis genes for the carotene production; (2) to produce oxygenated β-carotene derivatives by heterologous expression of the *crtW* gene (encoding β-carotene ketolase) of the marine *Agrobacterium aurantiacum*; (3) to integrate the *crtW* gene into the *Mucor* genome by different methods; (4) and to reveal the carotenoid spectra and to characterize the carotenoid production of some Zygomycetes in order to determine new producer strains potentially applicable in further analysis.

Transformation of fungal protoplasts was carried out by the polyethylene glycol-mediated method. Three different isoprenoid genes were involved in the study. Expression vectors that contained one of these genes driven either by their own promoter or by the regulator

sequences of the *Mucor* glyceraldehyde-3-phosphate dehydrogenase 1 gene (*gpd1*), were constructed. The *Mucor leuA* or *pyrG* genes were used as selection markers; they complement the leucine or uracil auxotrophy of the recipient *M. circinelloides* strain, respectively. Vectors were introduced alone ore in co-transformations to combine the isoprenoid genes. All transformants proved to be stable under selective conditions and some of them under non-selective conditions as well. Transformants were analyzed with hybridization and PCR techniques. Real-time PCR analysis revealed a relatively high copy number of the plasmids in the transformants and an unequal proportion of them in the co-transformants. Higher expression of the genes was also verified. The carotene production was analyzed by spectrofotometric, TLC and HPLC methods.

It has been found that *M. circinelloides* has β-carotene hydroxylase activity, therefore introducing the *crtW* gene may result in the production of several types of oxygenated β-carotene derivatives. Transformation with vector, containing the *crtW* gene under the control of *gpd1* promoter, was carried out (Papp et al. 2006) and co-transformations with the isoprene genes were also done. Changes in the carotenoid production due to expression of the *crtW* gene have been proven.

Integration the *crtW* gene into the *Mucor* genome was achieved by three different methods: homologous recombination with double crossing over, *Agrobacterium tumefaciens*-mediated transformation and restriction enzyme-mediated integration. The integration had been proven and analysed in several transformants by PCR, inverse-PCR, real-time PCR and hybridization techniques.

Carotene content of twenty one Zygomycetes strains was also analyzed. Some of them produced the same or higher amount of carotenoids than the wild type *M. circinelloides* or *B. trispora* strains. These strains were analyzed under different conditions, e.g. temperature, light and carbon source. For some of these strains, we started the development of new transformation systems that allows the direct selection of the transformants.

Papp T, Velayos A, Bartók T, Eslava AP, Vágvölgyi Cs, Iturriaga EA (2006) Heterologous expression of astaxanthin biosynthesis genes in *Mucor circinelloides*. Appl Microbiol Biotechnol 69:526-531.

Papp T, Nyilasi I, Csernetics Á, Galgóczy L, Vágvölgyi Cs (2008) Molecular studies on Zygomycetes fungi causing opportunistic infections. Rev Med Microbiol 19(2):39-46.

Supervisor: Tamás Papp
E-mail: csernetics.arpad@gmail.com

Increased genetic stability of a rationally designed reduced-genome *Escherichia coli*

Bálint Csörgő

Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

In an attempt to engineer a simplified, core-genome *Escherichia coli*, we have reduced the wild-type K-12 MG1655 genome by making surgically precise scarless deletions (Pósfai et al. 2006). Genome reduction was achieved without compromising the basic metabolic circuits of the cells. The new strains, with genomes up to 22% smaller, were designed by bioinformatic comparative genomics of four *E. coli* strains to identify non-essential genes and recombinogenic, mobile or cryptic virulence sequences, as well as genes with unknown functions for elimination.

These so-called multi-deletion strains (MDS) have several attractive properties which can make them useful in a wide variety of biotechnological applications. One of the most important of these properties is the increased genetic stability of these strains which includes an increase in both genomic and plasmid stability. This work focuses on the quantification of these different aspects of genetic stability. This was done using novel methods we developed for calculating mutation rates (Fehér et al. 2006) as well as rates of recombination within the cells.

Removal of all mobile genetic elements from the *E. coli* genome resulted in a lower mutation rate because of the lack of insertion events. In addition, the genes of three so-called error-prone DNA polymerases (*polB*, *dinB* and *umuDC*) were deleted resulting in a lower point-mutation rate. The resulting strain has a mutation rate that is close to one order of magnitude lower than the wild-type.

In addition to the increased fidelity of replication, lentiviral expression vectors harbored within different MDS strains proved to be more stable than in other commonly used cloning strains (Chakiath and Esposito 2007). By developing a plasmid-based system to measure recombination rates, we were able to quantify this improved stability. The most stable of our strains has a recombination rate that is over five times lower than the wild-type.

Chakiath CS, Esposito D (2007) Improved recombinational stability of lentiviral expression vectors using reduced-genome *Escherichia coli*. Biotechniques 43(4)1-3.

Fehér T, Cseh B, Umenhoffer K, Karcagi I, Pósfai G (2006) Characterization of *cycA* mutants of *Escherichia coli*. An assay for measuring in vivo mutation rates. Mutat Res 595:184-190.

Pósfai G, Plunkett G 3rd, Fehér T, Frisch D, Keil GM, Umenhoffer K, Kolisnychenko V, Stahl B, Sharma SS, de Arruda M, Burland V, Harcum SW, Blattner FR (2006) Emergent properties of reduced-genome Escherichia coli. Science 312:1044-1046.

Supervisor: György Pósfai
E-mail: csorgob@brc.hu

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Analysis of a structural motif in the membrane-associated [NiFe] hydrogenases

Emma Dorogházi

Department of Biotechnology, University of Szeged, Szeged, Hungary

Hydrogenases, catalyzing the following reaction: $2H^+ + 2e \Leftrightarrow H_2$, are harbored by numerous microorganisms. The cells dispose excess electrons through hydrogen production accomplished by hydrogenases, while consumption of the molecular hydrogen mostly provides electron source for various energy conserving processes, such as respiration. Sometimes, hydrogen can be the sole energy source for the cell growth. Hydrogenases are distinguished according to the metal content in their active center: they are classified as [NiFe], [FeFe] and [Fe] enzymes (Vignais et al. 2004). Minimally, a [NiFe] hydrogenase is composed of a large and a small subunit and they can be associated to the membrane or localized in the cytoplasm. The large subunit contains a binuclear metallocenter, while the small subunit hosting the Fe-S clusters, which conduct the electrons between the H_2-activating center and the surface of the protein.

Our model organism, the purple sulfur photosynthetic bacterium *Thiocapsa roseopersicina* BBS contains at least four active [NiFe] hydrogenases. The HynSL and the HupSL enzymes are attached to the cell membrane, while Hox1YH and Hox2YH are apparently localized in the cytoplasm (Kovacs et al. 2005).

There are several conserved motifs in the sequence of the hydrogenases, which are characteristic for these enzymes. These motifs have very important role for example in coordination of the metals of the active centre, in electron transfer, in interaction with other proteins or in translocation of the fully folded protein (Vignais et al. 2007).

We have noticed a highly conserved histidine-rich region with unknown function in the large subunit of [NiFe] hydrogenases. The HxHxxHxxHxH sequence occurs in the large subunit of all membrane-bound hydrogenases, but only two of these conserved histidines are present in the soluble hydrogenases.

In order to identify the function of this motif, mutant strains were made by site-directed point mutagenesis and their biochemical properties were characterized. The *in vivo* and *in vitro* activity measurements showed that the activity was influenced dramatically only in one of the mutants due to the replacement of the His residue with Ala. Nevertheless, this enzyme still remained in the membrane.

Western hybridization experiments were applied to investigate the proteolytic stability of the enzymes. It was found that the strongly reduced activity of the mutant hydrogenase could not be derived from the destabilization of the protein.

The oxygen sensitivity of the single amino acid mutant and the wild type protein was also compared for explaining the background of significant *in vitro* and *in vivo* activity loss in the mutant strain. The crude extracts from the wild type and mutant cells obtained in anaerobe box and on air was used for *in vitro* spectroscopic measurements, but the ratio of the activities of the wild type and the mutant protein was nearly the same independently on the environment of the cell destruction.

For further biochemical and biophysical investigations, large amount of enzymes have to be purified. First, a HynS-Strep-II Tagged fusion protein was constructed to purify the non-mutant HynSL enzyme by affinity chromatography. However the amount of the purified protein was not enough for further spectroscopic experiments and the specific activity of the Strep-II fused hydrogenase was lower as compared to the natural enzyme. We continued with the standard biochemical techniques and used fast protein liquid chromatography (FPLC) for purification of the wild type and mutant protein in large scale. These experiments are in progress and the first results are very auspicious.

For getting a complete picture about the structure-function relationship in HynSL hydrogenase, other conserved residues are being investigated, as well. From biotechnological point of view thermostability and oxygen tolerance of the hydrogenases are two crucial properties. In order to improve these properties of the enzymes, identification and functional characterization of candidate sequences potentially conferring these beneficial properties to the enzymes are to be done.

Kovács KL, Kovács AT, Maróti G, Mészáros LS, Balogh J, Latinovics D, Fülöp A, Dávid R, Dorogházi E, Rákhely G (2005) The hydrogenases of Thiocapsa roseopersicina. Biochem Soc Trans 33:61-63.
Vignais PM, Colbeau A (2004) Molecular biology of microbial hydrogenases. Curr Issues Mol Biol 6(2):159-188.
Vignais PM, Billoud B (2007) Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev 107:4206-4272.

Supervisors: Kornél L. Kovács, Gábor Rákhely
E-mail: doemma@brc.hu

Mechanistic insights into the role of translesion synthesis and its effect on genome stability

Himabindu Gali

Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

REV1 is a Y-family DNA polymerase. REV1 proteins contain a BRCT domain, which is important in protein-protein interactions. A suggested role for REV1 protein is as a scaffold that recruits DNA polymerases involved in translesion synthesis (TLS) of damaged DNA.

To elucidate the mechanism by which REV1 promotes DNA damage bypass, we have analyzed the progression of replication on ultraviolet light-damaged DNA in mouse embryonic fibroblasts that contain a defined deletion in the N-terminal BRCT domain of REV1, or that are deficient for REV1.

DNA fiber labeling method that has been previously described has been adapted in which two modified nucleotides IdU and BrdU were used to label newly replicated DNA. Incorporated IdU and BrdU were detected by fluorescent immunolabeling and the progression of replication fork was monitored. To examine the effect of UV damage to DNA on replication fork progression, cells were treated with either 20J/m^2 or 40J/m^2 UV dose at the end of first labeling period (IdU) and before second labeling (BrdU). Fork rates were calculated for each labeling period and the ratio of IdU to BrdU were analyzed. Under normal replication conditions ratio of IdU to BrdU is approximately 1. However an increase in this ratio directly corresponds to the rate of fork stalling during second labeling as a result of UV damage to DNA.

To investigate the role of REV1 BRCT and REV1 in replication fork progression, DNA fiber spreads were prepared, labeled forks were measured and compared to that of wild type cell line. In wild type cells with no UV treatment the average ratio of IdU to BrdU was 1.13 and REV1 BRCT and REV1 mutant cells showed an average ratio of 1.13 and 1.16 respectively. There was no significant difference in the rate of fork progression in any of the mutant cell lines as compared to the wild type line. Therefore, these genes are dispensable for the normal growth and viability of the cell.

The frequency of fork stalling in REV1 BRCT and REV1 mutant cell lines after 20J/m^2 or 40J/m^2 UV dose was measured. Both REV1 BRCT and REV1 mutant cells showed a significant increase in the ratio of IdU to BrdU in response to 20J/m^2 UV treatment and the ratios increased further at 40J/m^2 UV. In wild type cells with 20J/m^2 and 40J/m^2 UV treatment the average ratio of IdU to BrdU increased from 1.13 to 1.92 and 3.24. REV1 BRCT and REV1 mutant cells showed an increase in the ratio from1.13 to 4.14 and 4.99, 1.16 to 3.43 and 4.5 respectively. Furthermore, the exogenous expression of mREV1 in REV1 BRCT and REV1 mutant cell lines restored wild type phenotypes.

These results provide an evidence for the role of BRCT domain of REV1 in response to DNA damage and that REV1 plays a central role in replication fork progression of UV-damaged DNA.

Jansen J, Langerak P, Tsaalbi-Shtylik A, van den Berk PC, Jacobs H, de Wind N (2006) Strand-biased defect in C/G transversions in hypermutating immunoglobulin genes in Rev1-deficient mice. J Exp Med 203:319-323.
Jansen JG, Fousteri MI, de Wind N (2007) Send in the clamps: control of DNA translesion synthesis in eukaryotes. Mol Cell 28:522-529.
Merrick CJ, Jackson D, Diffley JF (2004) Visualization of Altered Replication Dynamics after DNA Damage in Human Cells. J Biol Chem 279(19):20067-20075.

Supervisor: Haracska Lajos
E-mail : hima@brc.hu

Uptake and degradation of xenobiotic in *Sphingomonas subarctica* SA1

Anna Gara

Department of Biotechnology, University of Szeged, Szeged, Hungary

Sulfanilic acid is a typical representative of sulfonated aromatic amines widely used and manufactured as an important intermediate in the production of azo dyes, plant protectives and pharmaceuticals. *Sphingomonas subarctica* SA1, a Gram-negative aerob bacterium is able to utilize sulfanilic acid as the only carbon, nitrogen, and sulfur source (Perei et al. 2001). In addition to sulfanilic acid, *Sphingomonas subarctica* SA1 could degrade six other aromatic compounds, such as sulfocatechol, protocatechol, para-amino benzoic acid, 3,5-dihydroxybenzoic acid and oil in soils.

Comparison of the protein patterns of cells grown on various substrates revealed that the strain used alternative metabolic pathways for biodegradation of these compounds. However, similar patterns were observed in the case of cells grown on sulfanilic acid and sulfocatechol. Therefore sulfocatechol is supposed to be formed, as first intermediate in the catabolism of sulfanilic acid. Unfortunately, sulfanilic acid could be converted by intact cells only but not by disrupted cells, thus the characterization of this reaction step was difficult. Nevertheless, sulfocatechol is further oxidized by a ring cleaving dioxygenase, named as sulfocatechol dioxygenase. This enzyme was partially purified and identified by mass spectrometry (Magony et al. 2007). A genomic locus harbouring the genes of sulfocatechol dioxygenase (*scaEF*) was also identified and upstream from these genes, few other *orfs* coding for proteins similar to muconate cycloisomerases (ScaA), lactone hydrolases (ScaB), maleylacetate reductases (ScaC) and an oxidase (ScaD) were recognized. These enzymes were actively overexpressed in *E. coli* and the sulfocatechol degradation pathway was reconstituted by the recombinant proteins.

The first step of sulfanilic acid degradation is not fully understood. The enzymes probably converting sulfanilic acid to sulfocatechol were very sensitive to cell disruption indicating that they were somehow related to the membrane. Proteomics approach was applied to identify of the enzymes catalyzing the sulfanilic acid conversion. Bands sepcifically appearing upon substrate induction in the membrane and soluble fractions were cut out and sequenced *de novo* by mass spectrometry.

The analysis of the proteomic data of the soluble fraction led to the identification of another gene set in the genome. In this locus, two genes likely coding for proteins involved in the oxidative deamination of sulfanilic were predicted.

Three specifically appearing membrane proteins were found in the membrane fractions of cells grown on sulfanilic acid. The aminotransferase is probably one component of the sulfanilic acid converting enzymes catalyzing the deamination of sulfanilic acid. It was shown to be poorly membrane associated, since it was also found in the soluble fractions. The second protein contained motifs of ATP-binding casettes indicating the energy-dependence of sulfanilic acid uptake. The third protein is a hypothetical TonB-dependent protein, which might play a role in many types of transport including iron uptake. The expression of the TonB-dependent protein is upregulated specifically by xenobiotics/aromatics and iron. Since, two enzymes of the degradation pathway are known to contain iron in their active center, it is plausible to assume, that the TonB dependent protein is involved in the iron transport to feed the extra iron demand of the enzymes taking part in the biodegradation.

From our data it is assumed that the uptake and conversion of sulfanilic acid is linked to a membrane protein complex and this association can function as a self-defending mechanism for the cell against the cytoplasmic occurrence of the toxic substrates. Furthermore, a potential link between the xenobiotics degradation and iron transport is suggested.

Magony M, Kákonyi I, Gara A, Rapali P, Perei K, Kovács KL, Rákhely G (2007) Overlaps between the various biodegradation pathways in Sphingomonas subarctica SA1. Acta Biol Hung 58:37-49.
Perei K, Rákhely G, Kiss I, Polyák B, Kovács KL (2001) Biodegradation of sulfanilic acid by Pseudomonas paucimobilis. Appl Microbiol Biotechnol 55:101-107.

Supervisor: Gábor Rákhely
E-mail: anna.gara@gmail.com

Role of salicylic acid pretreatment on the photosynthetic performance and the generation of reactive oxigen species and nitric oxide in tomato plants (*Solanum lycopersicum* Mill. L. cvar. Rio Fuego) under salt stress: acclimatization or programmed cell death

Katalin Gémes

Department of Plant Biology, University of Szeged, Szeged, Hungary

Salicylic acid (SA) has long been known as a signal molecule in the induction of defense mechanisms in plants (Raskin 1992) and it was shown to improve the acclimation to different abiotic stress factors, including high salinity (Szepesi et al. 2009). SA increased reactive oxygen species (ROS) production and therefore oxidative stress (Knörzer et al. 1999). SA was also shown to influence a number of physiological processes (Raskin 1992) e. g. inhibited the activity of Rubisco and thus the photosynthetic activity (Vernooij et al. 1994).

The aim of my work was to reveal, how ROS production ($O^{\cdot}$; H_2O_2) was modified by different concentrations of SA, how the SA-treated cells could acclimate to oxidative stress or why other tissues became committed to programmed cell death (PCD).

On the basis of the inhibity effect of SA on stomatal conductance and photosynthetic performance, which has been documented in several papers, it was presumed, that the ROS produced after SA pretreatment may be derived from an inhibited photosynthetic electron transport. This may also reduce the plants capacity to synthesise compatible osmolytes, such as sugars, during pretreatment or salt stress.

That is why we measured the changes in photosynthetic activity (chl*a* fluorescence induction parameters, CO_2 fixation rate as function of PAR or Ci and stomatal conductance) during pretreatment.

Tomato plants were grown hydroponically in the presence of different SA (10^{-3} M, 10^{-4} M, 10^{-7} M). Seven-week-old plants were exposed to 100 mM NaCl for a week.

Short-term pretreatment of plants with 10^{-3} M SA resulted in a permanent decrease in the stomatal conductivity and the CO_2 fixation rate compared to the control and also decreased the viability of plants. In contrast, after a transient decline photosynthetic parameters of plants grown in 10^{-7} and 10^{-4} M SA were not significantly different from the untreated control at the end of the pretreatment period. Salt stress also inhibited the photosynthetic activity, which was significantly alleviated by 10^{-4} M SA. The improved photosynthetic performance and the accumulation of soluble sugars as compatible osmolytes resulted in a partial osmotic adjustment and contributed to successful acclimation to high salinity in 10^{-4} M SA pretreated plants.

The accumulation of putrescine in the leaves and those of spermidine and spermine in the roots are adaptive feature of some halophyte species. We found similar changes in the polyamine spectrum of plants grown in 10^{-4} M SA at the end of pre-treatment period. Moreover these tissues produced less ethylene, a PCD inducing plant hormone, which coincided with higher viability of root apical cells.

As it was expected a significant accumulation of H_2O_2 occurred in the leaves and roots of plants exposed to 10^{-4}-10^{-7} M SA, but after three weeks the differences disappeared in the root tissues and remained in the leaves. We prepared mesophyll protoplasts as model system to investigate the effects of the compounds that accumulated in plants during pre-treatments on ROS production and to compare the results with intact plants.

Knörzer OC., Lederer B, Durner J, Böger P (1999) Antioxidative defense activation in soybean cells. Physiol Plant 107:294-302.
Raskin I (1992) Role of salicylic acid in plants. Ann Rev Plant Physiol And Plant Mol Biol 43:439-463.
Szepesi Á, Csiszár J, Gémes K, Horváth E, Horváth F, Simon LM, Tari I (2009) Salicylic acid improves the acclimation to salt stress by stimulating abscisic aldehyde

oxidase activity and abscisic acid accumulation, and increases Na^+ content in leaves without toxicity symptoms in Solanum Lycopersicum L. J Plant Physiol 166(9):914-925.

Vernooij B, Friedrich L, Morse A, Reist R, Kolditz-Jawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell 6:959-965.

Supervisor: Irma Tari
E-mail: gemeskatalin@freemail.hu

The role of Drosophila formin dDAAM in axon growth

Rita Gombos

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

In the developing nervous system, the growth cones have an essential role in guiding the axons to their targets. Directed growth cone motility in response to extracellular cues is produced by the coordinated regulation of peripheral F-actin and central microtubule networks. The peripheral F-actin is organized into long bundled actin filaments in the finger-like filopodia and diffuse networks of shorter actin filaments contained in the veil-like lamellipodia. Previous work identified many immediate regulators of F-actin dynamics in growth cones, and for some of these, it has been demonstrated that they act downstream of signaling pathways involved in axonal growth regulation. Key regulators of actin dynamics are the so called nucleation factors, such as the Arp2/3 complex and formins, which use different mechanisms to seed new actin filaments (Pak CW et al. 2008).

The formin proteins are involved in actin polymerization and growth by associating with the fast-growing end (barbed end) of actin filaments. Formins contain two homology domains the FH1, and the FH2. The actin nucleation-promoting activity of formins has been localized to the FH1-FH2 domains. The FH1 domain is a proline-rich region, that can bind the G-actin monomer carrying profilin protein. The FH2 domain is necessary and sufficient to nucleate actin in vitro (Goode BL and Eck MJ 2007).

The principal field of research in our group is the functional analysis of the single *Drosophila DAAM* ortholog. We revealed that *dDAAM* is transcribed pan-neurally from stage 11 of embryogenesis in the area of central nervous system (CNS). The immunostaining experiments demonstrated that dDAAM protein is highly enriched in neurites, where it shows a strong colocalization with actin. Therefore we wanted to investigate if this protein plays a role in neurite growth.

To examine the function of *dDAAM* in the CNS, we first carried out a loss of function (LOF) analysis by examining mutant embryonic ventral nerve cord and primary neuronal cultures. The mutant embryos showed strong CNS phenotypes, displayed frequent breaks in the connectives and commissures. The *dDAAM* mutant neurons were able to develop axons of similar length as their wild-type counterparts, but the filopodia of mutant neurons were reduced in number and in length. Thus, the LOF experiments suggest that *dDAAM* regulates filopodium formation in neuronal growth cones.

Next we examined the effect of the constitutively active form of dDAAM on the embryonic CNS. We detected that the overexpression of this form in the embryonic CNS results in severe fasciculation defects and embryonic lethality. To collect additional evidences that activated dDAAM has the potential to increase neurite number, we examined embryonic nerve cord cultures overexpressing this form. The ventral nerve cords expressing activated dDAAM exhibited a much denser neuritic meshwork and growed more extended axons than their wild type counterparts. In cultured primary neurons, the overexpression of activated protein increased the number of filopodia. These observations suggest that dDAAM plays a major role in the regulation of axonal growth, presumably by promoting actin assembly in the growth cone (Matusek et al. 2008).

In addition, we detected strong dDAAM expression in adult brain as well. In LOF analysis we found several axonal projection defects indicating a role in the formation of the adult neuropil.

To determine proteins that may act together with dDAAM in the regulation of axonal growth, we carried out a genetic interaction analysis. We demonstrated that dDAAM shows an interaction with *Ena and profilin*. Moreover, we identified Rac as the most likely activator of dDAAM in the developing nervous system. (Matusek et al. 2008).

Goode BL, Eck MJ (2007) Mechanism and function of formins in the control of actin assembly. Annu Rev Biochem 76:593-627.

Matusek T, Gombos R, Szécsényi A, Sánchez-Soriano N, Czibula A, Pataki C, Gedai A, Prokop A, Raskó I, Mihály J (2008) Formin proteins of the DAAM subfamily play a role during axon growth. J Neurosci 28(49):13310-13319.

Pak CW, Flynn KC, Bamburg JR (2008) Actin-binding proteins take the reins in growth cones. Nat Rev Neurosci 9(2):136-147.

Supervisor: József Mihály
E-mail: gombri@brc.hu

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Biohydrogen production using the cellulose containing plant biomass

Zsófia Herbel

Department of Biotechnology, University of Szeged, Szeged, Hungary

There are many serious gobal problems caused by mankind in the last dacades. Among these, two have oustanding importance: a) the enviromentally friendly, biological degradation of the large amounts of organic waste poduced by the industrial sector; b) as well as the reinforcement of the energy supply by renewable energy source and the substitution of the current energy carriers by environmentally sound fuels. Hydrogen is considered as the best candidate for the future energy carriers, since just pure water is formed during its oxidation. There are biological tools for producing hydrogen and hydrogen evolving photosynthetic or fermentative microbes are primarily involved in these processes. In the dark fermantative processes, usually biopolymers of agricultural origin used to be the substrate, which have to be first converted to simpler monomers being fed to the hydrogen producing bacteria. Strains capable to convert and utilize complex biopolymers are of extrme importance. One of the best candidates is the Gram positive, hyperthermophilic, anaerobic *Caldicellulosiruptor saccharolyticus.* (Bagi et al. 2007) Its biotechnological importance is that it is capable to degrade cellulose-based biomasses, such as paper, or energy plants (which are found in the large quantities) and has hydrogenases for removal of excess electrons formed during the fermentative metabolism. The genome of this bacterium is available and the strain was shown to possess not only numerous glycosidases required for the hydrolysis of different kinds of polysaccharides, but also hydrogenase enzymes responsible for hydrogen production.

In our group an enviromentally friendly biological method have alredy been developed by wich we waste of animal origin could be transformed to hydrogen. (Bálint et al. 2005.)

In my work I aim at adapting this process to cellulose-based waste of plant origin. Morover,on the basis of the known genome of this organism, I intend to create a genetically modified strain which is capable of degrading the avaible biomass (currently filter paper) more efficiently and thereby producing significantly higher amounts of hydrogen.

According to our aims, I studied the hydrolysis of untreated filter paper in batch fermentation conditions in the presence of six various kinds of sugar using minimal media containing no other carbon sources. In paralel, I monitored the hydrogen evolution from sugars and filter paper alone and various combinations.

My results clearly showed, that *C. saccharolitycus* was able to degrade the untreated filter paper and to produce abundance hydrogen from this material in the presence of minimal amount of sugar. From the sugar specificity, it is likely that these sugars are basically necessary for the induction of cellulase enzymes., thus paper decomposition can be promoted by sugars.

For better understanding the molecular background of the events and for further improvement of the process genetically modified strains should be constructed. However, no protocol is available for gene transfer into *C. saccharolyticus*. In order to introduce foreign DNS into the cells, a functional genetic system has to be developed. As a first step, the efficient plating technique was ameliorated and numerous suitable vectors (replicative and non replicative) were collected and constructed to achieve sustainable vector replication or to modify the given part of the genome. Furthermore the development of an efficient method for introduction of foreign DNA is also requisite.

My future plans are the optimization of the paper decomposition conditions, getting deeper insight into the the molecular mechanism of the paper/sugar metabolism → hydrogen conversion.

Bagi Z, Acs N, Bálint B, Horváth L, Dobó K, Perei KR, Rákhely G, Kovács KL (2007) Biotechnological intensification of biogas productin. Appl Microbiol Biotecnol 76:473-482.

Bálint B, Bagi Z, Tóth A, Rákhely G, Perei K, Kovács KL (2005) Utilization of keratin-containing biowaste to produce biohydrogen. Appl Microbiol Biotecnol 69:404-410.

Supervisors: Gábor Rákhely, Kornél L. Kovács
E-mail: herbelzs@brc.hu

Construction and characterization of synthetic genetic oscillator in yeast

Anita Kapus

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

The design of gene expression systems with both spatial and temporal regulation has been an area of intense scientific interest during the last ten years. Our aim is to create a synthetic genetic circuit in yeast based on transcriptional – translational feedback loops mimicking the structure (input, oscillator, output) and function (self-sustaining oscillation and resetting) of eukaryotic circadian clocks. The circuit will serve as an ideal test system for mathematical modeling describing oscillatory mechanisms (eg. circadian clocks), since all components are known, well characterized and can be easily modified/adjusted in order to test predictions from the model. The main requirements for such a system are: a genetic network with well defined components, which do not interfere with the physiology of yeast; option to set/modify the expression level/turn-over rates of components, proper input/resetting mechanism and easily measurable output (in vivo, real-time).

First we created and tested components for the input pathway of the oscillator. In the circadian systems of eukaryotes, external signals (e.g. light and/or temperature) reach the oscillating genetic network through the input pathway and cause an acute change in the expression

or stability of one or more oscillator components, which consequently resets the phase of the oscillation. In our system, light act as a resetting signal via an artificial light switch. The switch is based on the light-dependent interaction between the plant photoreceptor phytochrome A (PHYA) and its specific interacting protein FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) [Hiltbrunner, 2005]. The activator and the DNA-binding domain of the GAL4 transcription factor are fused to FHY1 and PHYA, respectively, so transcription from GAL4-dependent promoters is activated only by the physical interaction between PHYA and FHY1 in mutant yeast cells lacking the endogenous GAL4 protein. Red light converts PHYA to its active form, which interacts with FHY1; however, far-red light diminishes the interaction, because it converts PHYA to its inactive conformer. To test the function of the light switch we measured expression of the GAL1 promoter driven luciferase reporter gene (GAL1:LUC+). We showed that luciferase activity is tightly controlled by red or far-red light pulses indicating the proper function of the light switch.

The core components of the oscillator (termed "Yeascillator") are represented by two artificial genes whose gene products can mutually regulate transcription of each other. Expression of the positive protein [Gari, 1997] is driven by a modular promoter, which contains cis-elements for copper induction and for binding of the negative component protein. The basal activity of this promoter is controlled by copper in the media. The positive protein is a fusion between the tetracycline-responsive transactivator (tTA) and the YFP proteins. tTA-YFP is able to bind to a specific cis-element (*tetO*) built in the promoter of the second gene encoding the negative protein. Binding of tTA-YFP can be controlled by doxycycline and results in the activation transcription. The negative protein consists of the DNA-binding domain of the bacterial LexA protein, the yeast transcriptional repressor SSN6 and the CFP proteins. LexA-CFP-SSN6 binds to the modular promoter of the first gene via specific LexA binding sites and represses transcription. The different fluorescent protein tags (YFP and CFP) allow simultaneous detection and quantification of the positive and negative protein components. The output of the oscillator is represented LUC+ reporter gene controlled by a promoter, which responds to the positive component only. Our preliminary results indicate that the two genes can regulate each other as expected, but detection of oscillation will require more optimizations.

Garí E, Piedrafita L, Aldea M, Herrero E (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. Yeast 13:837-848.

Hiltbrunner A, Viczian A, Bury E, Tscheuschler A, Kircher S, Toth R, Honsberger A, Nagy F, Fankhauser C, Schafer E (2005) Nuclear accumulation of the phytochrome A photoreceptor requires FHY1. Curr Biol 15:2125-2130.

Supervisors: Laszlo Kozma-Bognar and Ferenc Nagy
E-mail: kapusa@brc.hu

Regulation of single spike initiated feed-forward networks through 5-HT-2 receptors in the human and rat cerebral cortex

Gergely Komlósi[1], Szabolcs Oláh[1], Gábor Molnár[1], Miklós Füle[1], Noémi Molnár[1], Pál Barzó[2], Gábor Tamás[1]

[1]Research Group for Cortical Microcircuits of the Hungarian Academy of Sciences, University of Szeged, Szeged, Hungary; [2]Department of Neurosurgery, University of Szeged, Szeged, Hungary

The performance of the human cerebral cortex is unparalleled by the nervous system of other species and this is presumably supported by refined, but largely unknown features of the human microcircuit. We have shown that single action potentials in pyramidal cells can trigger reliable and stereotyped series of multiple postsynaptic potentials in simultaneously recorded pyramidal cells and interneurons in the human cerebral cortex. These polysynaptic event series are composed of alternating excitatory and inhibitory postsynaptic potentials lasting up to tens of milliseconds (Molnar, Olah et al. 2008).

We tested how these complex network events could be affected by the endogenous neurotransmitter serotonin known to be involved in several physiological processes, and implicated in many psychiatric disorders (Jones and Blackburn 2002). We recorded from pairs, triplets and quadruplets of neurons in slices of human association cortices looking for mono- and polysynaptic connections. Nanomolar concentrations of serotonin reversibly supressed single pyramidal spike activated di- and polysynaptic events, and this effect could be mimicked by the 5-HT-2 receptor agonist alpha-methylserotonin. Similarly, alpha-methylserotonin was effective in eliminating axo-axonic cell triggered polysynaptic but not disynaptic events.

We then investigated the effect of serotonin on monosynaptic unitary connections between various types of layer 2/3 neurons. We found that serotonin and alpha-methylserotonin decreased the amplitude of EPSPs between pyramidal cells and from pyramidal to various types of interneurons including fast-spiking basket and axo-axonic cells but little or did not change the amplitude of IPSPs from fast-spiking to pyramidal neurons.

To examine the mechanism by which serotonin might modulate excitatory transmission, we analysed the percentage of failures to evoke an EPSP and the coefficient of variation of unitary EPSP amplitudes with and without serotonin and alpha-methylserotonin. Both serotonin and alpha-methylserotonin increased the failure rate and the coefficient of variation suggesting a presynaptic site of modulation of serotonin in the glutamatergic synaptic transmission.

Finally, we found that therapeutic concentrations of the serotonin reuptake inhibitor fluoxetine, a widely prescribed medication for treatment of depression, could enhance the effect of serotonin on excitatory synaptic transmission.

In conclusion, activation of 5-HT2 receptors can eliminate pyramidal cell activated feed-forward network events presumably via down-regulation of glutamate release probability in pyramidal axon terminals.

Jones BJ, Blackburn TP (2002) The medical benefit of 5-HT research. Pharmacol Biochem Behav 71(4):555-568.
Molnár G, Oláh S, Komlósi G, Füle M, Szabadics J, Varga C, Barzó P, Tamás G (2008) Complex events initiated by individual spikes in the human cerebral cortex. PLoS Biol 6(9):e222.

Supervisor: Gábor Tamás
E-mail: komgeri@bio.u-szeged.hu

Identification of potential mycotoxin producing *Fusarium* species in Hungarian wheat grain samples

Zsuzsanna Koncz

Eszterházy Károly College, EGERFOOD Regional Knowledge Centre, Eger, Hungary

Fusarium Head Blight (FHB) is a disease complex of cereals, in which several fungal species may cause symptoms. The species found as the major cause of head blight of wheat are *F. graminearum* and *F. culmorum*. Less frequently isolated species are *F. acuminatum*, *F. avenaceum*, *F. poae* and *F. sporotrichioides*. FHB can significantly reduce grain yield and quality.

Fusarium species are known as mycotoxin producers. The most predominant mycotoxins found in small-grain cereals are 8-ketotrichothecenes such as deoxynivalenol (DON) and nivalenol (NIV) and their acetylated derivatives including 3-acetyldeoxynivalenol (3-ADON) and 15-ADON, as well as an oestrogenic mycotoxin, zearalenone (Mirocha et al. 1989). A less frequently examined mycotoxin group is of the enniatins (ENs). *F. avenaceum*, *F. poae*, *F. sporotrichioides* and *F. tricinctum* are the main sources of ENs (Nicholson et al. 2004, Ivanova et al. 2006).

Species identification of mycotoxin-producing *Fusarium* species is of high importance in relation to FHB. The polymerase chain reaction (PCR) is a useful technique for the identification and differentiation of *Fusarium* species.

The aim of this study was to apply species-specific PCR-based assay for the identification of *Fusarium* species from Hungarian wheat grains. After processing 75 wheat samples of different geographical origin we isolated 255 *Fusarium* strains. Identification with species-specific PCR primers revealed the following species distribution: *F. acuminatum* 7.5 %, *F. avenaceum* 8.5 %, *F. graminearum* 37.5 %, *F. poae* 30.5 % and *F. sporotrichioides* 16 %. The results were confirmed by morphological identification after culturing the isolates on potato dextrose agar plates.

In addition to the species identification, we also performed PCR reactions to reveal the presence/absence of genes responsible for the production of several toxins (DON, 3-ADON, 15-ADON, NIV and ENs) in the *Fusarium* isolates. *F. graminearum* proved to be the most important fungus responsible for different diseases of small-grain cereals in Hungary. We used diagnostic primer sets, based on the *Tri3* (3-ADON and 15-ADON), *Tri5* (DON) and *Tri7* (NIV) trichothecene genes (Qurta et al. 2006), in multiplex PCR for the detection of *F. graminearum* chemotypes. An additional primer set was used to detect the *esyn1* gene (Kulik et al. 2007) for the detection of potential enniatin-producing *F. avenaceum*, *F. poae* and *F. sporotrichioides* species.

The investigated 96 *F. graminearum* isolates were potential producers of both DON and NIV toxins. The chemotypes were the following: DON 1 isolate, DON-NIV 1 isolate, DON-15-ADON 94 isolates. There was no isolate found containing the gene responsible for 3-ADON production. Our results suggest that strains of *F. graminearum* prevailing in Hungarian wheat-growing regions belong mainly to the DON-15-ADON chemotype.

Each of the examined *F. avenaceum*, *F. poae* and *F. sporotrichioides* isolates were positive for the *esyn1* gene, except two *F. poae* isolates. This is the first data set of enniatin-producing potential of Hungarian *Fusarium* isolates and shows that toxic potential of these strains may be underestimated.

Ivanova L, Skjerve E, Eriksen GS, Uhlig S (2006) Cytotoxicity of enniatins A, A1, B, B1, B2 and B3 from *Fusarium avenaceum*. Toxicon 47:868-876.
Kulik T, Pszczótkowska A, Fordonski G, Olszewski J (2007) PCR approach based on the *esyn1* gene for the detection of potential enniatin-producing *Fusarium* species. Int J Food Microbiol 116:319-324.
Mirocha CJ, Abbas HK, Windels CE, Xie W (1989) Variation in deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone production by *Fusarium graminearum*. Appl Environ Microbiol 55:1315-1316.
Nicholson P, Simpson DR, Wilson AH, Chandler E, Thomsett M (2004) Detection and differentiation of trichothecene- and enniatin-producing *Fusarium* species on small-grain cereals. Eur J Plant Pathol 110:503-514.
Quarta A, Mita G, Haiclukowski M, Logrieco A, Mulé G, Visconti A (2006) Multiplex PCR assay for the identification of nivalenol, 3- and 15-acetyl-deoxynivalenol chemotypes in *Fusarium*. FEMS Microbiol Lett 259:7-13.

Supervisors: János Varga, Árpád Szécsi
E-mail: koncz.zsu@gmail.com

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Phosphatidylglycerol is important in the assembly and function of PSII reaction center

Hajnalka Laczkó-Dobos

Department of Plant Biology, Biological Research Center, Hungarian Academy of Sciencies, Szeged, Hungary

Phosphatidylglycerol (PG) is a ubiquitous anionic phospholipid in almost all organisms. The structural and functional roles of anionic lipids in photosynthesis have raised scientific interest for a long time. The role of PG in photosynthetic organisms has previously been studied using either biochemical or molecular genetic approaches. The recent identification of genes encoding enzymes required for the biosynthesis of PG in cyanobacteria and eukaryotic plants, and the subsequent generation of mutants defective in the biosynthesis of PG, has provided powerful molecular tools to understand the function of PG in photosynthetic organisms. The role of PG has been extensively studied in two PG-less mutant strain of *Synechocystis sp.* PCC6803: Δ*pgsA* (Hagio 2000) and Δ*cdsA* (Sato 2000). Previously it was demonstrated that PG is required for the formation and function of thylakoid membranes in cyanobacteria and plants (Wada and Murata 2007; Domonkos 2008).

In the present investigation we constructed and characterized a new PG deficient mutant of *Synechocystis sp.* PCC6803. We inactivated the *cdsA* gene in phycobiliproteinless mutant, PAL, which compensates the missing light harvesting complex by high cellular content of PSII (Ajlani 1998). The PAL/Δ*cdsA* mutant provided a unique experimental system for a more detailed study of the role of PG in PSII function/assembly. We analyzed the influence of PG depletion on the fluorescence induction, thermoluminescence, biosynthesis and assembly of PSII protein subunits. The mutant cells grew only in a medium supplemented with PG. Depletion of PG in the cells resulted (i) in an inhibition of cell growth/division, (ii) in a small change in pigment composition, (iii) in the inactivation of oxygen evolution, (iv) in a modification of the fluorescence induction curve that pointed to some damage of Q_B, but not the donor side, (v) in a modification of the TL glow curve to give only shifted Q-band which is an indicator for suppression of electron transfer between Q_A and Q_B, and it does not affect the redox levels of Q_A and S_2. Two-dimensional PAGE showed that in the absence of PG (a) PSII dimer was monomerised, and (b) the CP43 protein was detached from a major part of the PSII core complex. [35S]-methionine labeling confirmed that PG depletion did not block de novo synthesis of PSII proteins. We conclude that PG is required for the binding of CP43 within the PSII core complex (Laczko-Dobos 2008). This is in good agreement with the presence of a PG molecule localized between D1 and CP43 subunits by X-ray crystallographic structure of *Thermosynecococcus elongatus* (Guskov 2009).

Ajlani G, Vernotte C (1998) Construction and characterization of a phycobiliprotein-less mutant of Synechocystis PCC6803, Plant Mol Biol 37:577–580.

Domonkos I, Laczkó-Dobos H, Gombos Z (2008) Lipid-assisted protein-protein interactions that support photosynthetic and other cellular activities. Prog Lipid Res 47(6):422-435.

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A, Saenger W. Cyanobacterial photosystem II at 2.9-Ångresolution and the role of quinones, lipids, channels and chloride. Nature structural and molecular biology 16(3):334-342.

Hagio M, Gombos Z, Varkonyi Z, Masamoto K, Sato N, Tsuzuki M, Wada H (2000) Direct evidence for requirement of phosphatidylglycerol in photosystem II of photosynthesis, Plant Physiol 124:795–804.

Laczko-Dobos H, Ughy B, Toth SZ, Komenda J, Zsiros O, Domonkos I, Parducz A, Bogos B, Komura M, Itoh S, Gombos Z (2008) Role of phosphatidylglycerol in the function and assembly of Photosystem II reaction center, studied in a cdsA-inactivated PAL mutant strain of *Synechocystis sp.* PCC6803 that lacks phycobilisomes. Biochim Biophys Acta 1777:1184-1194.

Sato N, Hagio M, Wada H, Tsuzuki M (2000) Requirement of phosphatidylglycerol for photosynthetic function in thylakoid membranes, Proc Natl Acad Sci USA 97:10655–10660.

Wada H, Murata N (2007) The essential role of phosphatidylglycerol in photosynthesis, Photosynth Res 92:205–215.

Supervisor: Zoltán Gombos
Email: dobosh@gmail.com

Characterization of catalase genes in *Rhizopus oryzae*

Beáta Linka

Department of Microbiology, Faculty of Sciences and Informatics, University of Szeged, Szeged, Hungary

Zygomycosis is a diverse group of mycotic diseases caused by members of the class Zygomycetes. The main risk factors are diabetic ketoacidosis; cancer and its therapy; solid organ or bone marrow transplantations; prolonged steroid use; neutropaenia; deferoxamine treatment to manage iron overload and burn injuries (Papp et al. 2008). Thermophilic members of the genus *Rhizopus*, especially *R. oryzae*, are considered as the main causative agents of zygomycoses. During the past decades, such infections have emerged in an increasing number due to the widespread use of immunosuppressive therapy, intensive cancer chemotherapeutic regimens and broad-spectrum antimicrobial agents. High mortality rates, difficulties in the diagnosis and resistance to the most widely used antifungal drugs are characteristic features of zygomycoses underlying the importance of this fungal group (Ribes et al. 2000). All these aspects indicate that development of new strategies to prevent and treat these infections is urgently needed.

The aim of our study is identification and analysis of the genetic background of the virulence of opportunistic pathogen Zygomycetes.

Generation of oxidative products by phagocytic cells is known to be one of the important host defence mechanisms directed towards the killing of invading microorganisms (Gallin at al. 1993). Catalases may provide protection against reactive oxygen species produced by neutrophile granulocytes of the human immune system (Chang et al. 1998). Neutropenia is a considerable risk factor of zygomyçoses. In this study, catalase encoding genes of *R. oryzae* have been isolated, and their functional analysis has been started.

Four possible catalase genes were found in the *R. oryzae* genome database (*Rhizopus oryzae* Sequencing Project) by similarity searches with known fungal catalases. These genes and their adjacent regions were amplified by PCR from the genomic DNA of *R. oryzae* and cloned into the vector pBluescriptII SK+ (Stratagene). To reveal their function and to investigate their possible role in the pathogenicity, deletion mutants were created in the case of each isolated genes. Four vectors suitable to create deletions in the different genes were constructed; in each vector, the *pyrG* gene of *R. oryzae* encoding orotidine-5'-monophosphate decarboxylase was placed between the 5' and 3' flanking regions of the appropriate catalase genes. To ensure double crossover gene replacement, linear fragments were cut from the plasmids and used to transform protoplasts of a uracil auxotrophic *R. oryzae* strain using the polyethylene glycol-mediated method.

Integration of the transferred DNAs into the host genome and deletion of the appropriate catalase genes was proven by PCR and Southern blot analysis. Catalase activity of the recipient strain and the four mutants constructed were *in vitro* tested. Effect of hydrogen peroxide on the fungal growth was examined on agar plates and in a microtiter plate assay. All four catalase genes proved to be functional. In all types of mutants, deletion of a catalase gene increased markedly the sensitivity of the transformants to hydrogen peroxide. The strain deficient in the gene designated as 16995 was the less susceptible to hydrogen peroxide whereas the strains deficient in the other genes proved to be more sensitive. Further gene expression studies with isolated genes are in progress and we also plan to use the constructed deletion mutants in pathogenicity tests.

Chang YC, Segal BH, Holland SM, Miller GF, ÉS Kwon-Chunn KJ (1998) Virulence of Catalase-deficient *Aspergillus nidulans* inp47phox-/-,Mice. J Clin Invest 101:1843-1850.

Gallin JI, Buescher ES, Seligmann BE, Nath J, Gaither T, Katz P (1983) NIH conference. Recent advances in chronic granulomatous disease. *Ann. Intern. Med.* 99:657–674.

Papp T, Nyilasi I, Csernetics Á, Galgóczy L, Vágvölgyi Cs (2008) Molecular studies on Zygomycetes fungi causing opportunistic infections. *Rev Med Microbiol* 19:47-55.

Rhizopus oryzae Sequencing Project, Broad Institute of Harvard and MIT 2004, http://www.broad.mit.edu

Ribes JA, Vanover-Sams CL, Baker DJ (2000) Zygomycetes in Human Disease. Clin Microbiol Rev 13:236-301.

Supervisor: Tamás Papp
E-mail: bealinka@gmail.com

Comprehensive genetic and biochemical examination of the polyubiquitin receptors in *Drosophila melanogaster*

Zoltán Lipinszki

Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

The ubiquitin-proteasome system is responsible for the polyubiquitilation and selective degradation of damaged, misfolded and short-lived regulatory proteins to ensure the proper homeostasis of the eukaryotic cell. Recognition of polyubiquitinated substrates by the proteasome is a highly regulated process that requires polyubiquitin receptors (p54/Rpn10: proteasome receptor subunit; Dsk2 and Rad23: non-proteasomal receptors). The mechanism of substrate recognition and delivery to the proteasome is well known in single cell eukaryotes (e.g. yeast), but unresolved in Metazoans.

We found that the subunit composition of the regulatory particle (RC) of the *Drosophila* 26S proteasome changes in a developmentally regulated fashion (Lipinszki et. al. submitted manuscript). The concentration of the p54/Rpn10 subunit falls suddenly at the end of embryogenesis, remains low throughout the larval stages, starts to increase again in the late third instar larvae and remains high in pupae and adults. A similar developmentally regulated fluctuation could be observed in the concentrations of the Rad23 and Dsk2 extraproteasomal polyubiquitin receptors. Our *in vitro* experiments revealed that protein extracts of first or second instar larvae can selectively degrade the embryonic p54/Rpn10 subunit of the 26S proteasome and the Dsk2 and Rad23 polyubiquitin receptors; whereas all the other tested proteins remained intact. The above observations and the fact that the gene expressions of the receptors remain constant during the development suggest that a selective protease is activated during the early larval stages. We successfully purified and identified this protease. Moreover, all the three receptors carry an extended intrinsically unstructured segment within the molecule, which can be the hot spot for the regulator protease.

To follow the *in vivo* fate of subunit p54/Rpn10, transgenic *Drosophila melanogaster* lines encoding the N-terminal half (NTH), the C-terminal half (CTH) or the full-length p54/Rpn10 subunit have been established in the inducible Gal4-UAS system. The daughterless-Gal4 driven whole-body expression of the full-length subunit or its NTH did not produce any detectable phenotypic changes and the transgenic products were incorporated into the 26S proteasome. The transgene-encoded CTH was not incorporated into the 26S proteasome, caused third instar larval lethality and it was found to be multiubiquitinated. This modification, however, did not appear to be a degradation signal, the half-life of the CTH is over 48 hours. The accumulation of the CTH disturbed the developmentally regulated changes of the subunit composition of the RP and, interestingly, the emergence of the selective proteolytic activity responsible for the depletion of the polyubiquitin

receptors. The accumulation of CTH also suspended the MG132 insensitive (proteasome inhibitor), but PMSF (serine-type endopeptidase inhibitor) sensitive proteolytic degradation of the p54/Rpn10, Dsk2 and Rad23 during the early larval stages.

Interestingly, CTH carrying three active UIM sequences extra-proteasomally traps the Dsk2 protein, hindering its interaction with the 26S proteasome. Our *in vitro* and *in vivo* studies revealed that in *Drosophila* UIM motifs of p54 can selectively bound the N-terminal UBL (ubiquitin like) domain of Dsk2. We suppose that contrary to the yeast model in which Rpn1 and Rpn2 scaffold subunits of the RC anchor Dsk2, Rad23 and Ddi1, in *Drosophila* the major polyubiquitin receptor Dsk2 (Lipinszki et. al. manuscript in preparation) docs to the C-terminally localized UIMs of the p54. Nevertheless, is has been demonstrated that p54 is a shuttling subunit of the proteasome (Kiss et. al., Szabó et. al.). It is conceivable that under regulation (e.g. ubiquitilation) p54 dissociates from the proteasome, and forms a heteromer with the Dsk2/substrate dimer, which is followed by the reassociation of the whole complex to the proteasome for degradation of the substrate protein.

Kiss P, Szabó A, Hunyadi-Gulyás E, Medzihradszky KF, Lipinszki Z, Pál M, Udvardy A (2005) Zn2+-induced reversible dissociation of subunit Rpn10/p54 of the Drosophila 26 S proteasome. Biochemical J (2005) 391, 301-310.

Lipinszki et al. (Submitted manuscript; JCS)

Szabó Á, Pál M, Deák P, Kiss P, Újfaludi Zs, Pankotai T, Lipinszki Z, Udvardy A (2007) Molecular characterization of the Rpt1/p48B ATPase subunit of the *Drosophila melanogaster* 26S proteasome. Mol Genet Genomics 278(1):17-29.

Supervisor: Andor Udvardy
e-mail: lzoltan@brc.hu

Neuroprotection in ischemic adult rat brain

Máté Marosi

Department of Physiology, Anatomy and Neuroscience, University of Szeged, Szeged, Hungary

Transient global ischemia elicits selective, delayed neuronal death. If the ischemia is short, neuronal damage occurs only in vulnerable areas (Pulsinelli et al. 1985). The pyramidal neurons in the hippocampal CA1 region are very vulnerable. Global ischemia impairs memory and learning functions. It is widely accepted that activation of the excitatory amino acid receptors plays an important role in neuronal death in stroke (Choi 1988). It has recently been reported that glutamate-induced excitotoxicity and a cellular calcium overload are among the key factors of cell death in brain ischemia, especially in the gray matter. By definition, excitotoxicity is a result of overexcitation of the glutamate receptors. In turn, neuroprotective strategies have utilized antagonists of the glutamate receptors to prevent excitotoxic neuronal loss.

The neuroprotective effect of L-kynurenine sulfate (KYN) was studied. KYN pretreatment decreased the number of injured pyramidal cells in the CA1 region of the hippocampus in the four-vessel occlusion (4VO)-induced ischemic adult rat brain. KYN post-treatment proved to be much less effective. In parallel with the histology, a protective effect of KYN on the functioning of the CA1 region was observed: long-term potentiation (LTP) was abolished in the 4VO animals, but its level and duration were restored by pretreatment with KYN. It is concluded that the administration of KYN elevates the KYNA concentration in the brain to neuroprotective levels (Sas et al. 2008).

The excess Glu which causes neuronal death via excitotoxicity, is normally controlled by members of a family of Na^+-dependent Glu transporters. By pumping Glu, they guarantee the presence of Glu in brain fluids at levels at which its exerts neither excitotoxic nor unsolicited excitatory effects. Glu transporters located on the brain vasculature may also play an important role in controlling extracellular Glu levels via a brain-to-blood Glu efflux. The scavenging of blood Glu increases the driving force for the brain-to-blood Glu efflux and causes a decrease of the excess Glu present in the brain. (Teichberg et al. 2008)

In the second series of experiments we evaluated the effects of the blood glutamate scavenger oxaloacetate on the impaired LTP observed in the rat 2-vessel occlusion ischemia model. Transient incomplete forebrain ischaemia was produced 3 days before LTP induction. Although the short transient brain ischaemia did not induce histologically identifiable injuries, it resulted in an impaired LTP function in the hippocampal CA1 region without damaging the basal synaptic transmission between the Schaffer collaterals and the pyramidal neurons. This impairment could be fended off in a dose-dependent manner by the i.v. administration of oxaloacetate immediately after the transient hypoperfusion. These results suggest that oxaloacetate-mediated blood and brain glutamate scavenging contributes to the restoration of the LTP after its impairment by brain ischaemia. (Marosi et al. 2009)

Our results suggest that both agents have potential clinical usefulness for the prevention of neuronal loss in stroke conditions.

Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1:623-634.

Marosi M; Fuzik J; Nagy D; Rákos G; Kis Z; Toldi J; Teichberg VI; Ruban-Matuzani A; Farkas T (2009) Oxaloacetate restores the long-term potentiation impaired in rat hippocampus CA1 region by 2-vessel occlusion. Eur. J. Pharm. 604:51-57.

Pulsinelli WA (1985) Selective neuronal vulnerability: morphological and molecular characteristics. Prog. Brain Res. 63:29–37.

Sas K, Robotka H, Rozsa E, Agoston M, Szenasi G, Gigler G, Marosi M, Kis Z, Farkas T, Vecsei L, Toldi J, (2008) Kynurenine diminishes the ischemia-induced histological and electrophysiological deficits in the rat hippocampus. Neurobiol Dis. 32:302–308.

Teichberg VI, Cohen-Kashi-Malina K, Cooper I, Zlotnik A, (2009). Homeostasis of glutamate in brain fluids: an accelerated brain-to-blood efflux of excess glutamate is produced by blood glutamate scavenging and offers protection from neuropathologies. Neuroscience 158(1):301-308. Epub 2008 Mar 18.

Supervisor: Tamás Farkas
E-mail: marosi.mate@gmail.com

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Functional characterization of candidate genes in barley: transgenic plants and grown cultivars

Bettina Nagy

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Barley (*Hordeum vulgare L.*) is one of the major and most distributed crops in the world. Currently it is becoming a novel cereal model plant representing a number of small-grain cereal species. While the barley genome is similar to that of other cereals, it is amenable to explorations of molecular genetics through its true diploidy.

The first Agrobacterium-mediated barley transformation reports was published in 1997 by Tingay and co-workers, using the variety called Golden Promise. The method that we established here was developed for this model cultivar and it's based upon protocols by Trifonova et al. (2001) and Kumlehn (IPK, Gatersleben, unpublished).

The selected genes we used for the plant transformations can be divided into the following three subgroups:

1. The regulators of the cell divison cycle: *MsCDKB2;1* (a *Medicago sativa* cyclin dependent kinase which plays a central role in regulation of the cell cycle, in particular in the G2/M phase transition and in mitosis). In previous studies it revealed that the overproduction of the *MsCDKB2;1* resulted in significant changes in agronomically important parameters in transgenic rice (Lendvai et al., unpublished). Other genes of this group, like OsPP2A B" regulatory subunit, OsRBRI2, OsRBRI5 are previously identified interactors of rice retinoblastoma-related protein, OsRBR1. Since cell cycle regulatory functions of the retinoblastoma proteins are primarily modulated by changing their phosphorylation status, *in planta* studies of the OsRBR1 interaction partner, the OsPP2A protien phosphatase B" regulatory subunit is particularly important from this viewpoint.

2. The 'oxidative stress-defense genes'. First transformation from this group of genes were made by the alfalfa aldo-keto reductase, *MsALR*. This enzyme plays important role in detoxification of the reactive aldehydes issued during oxidative stress, and helps the recovery of the plants (Oberschall et al. 2000). In order to accumulate protective enzymes in different subcellular compartments we constructed a vector for chloroplast targeting of protective enzymes using the transit peptide encoding region of the barley Rubisco LSU gene.

3. The genes involved in grain size determination (*GW2*, *GIF1*). Loss of *GW2* function increased grain width, weight and yield (Song et al. 2007) Antisense approach results increased grain size, even with constitutive expression of gene fragment in transgenic rice. We have identified and cloned the homologous gene from barley, a specific fragment of it was used for the generation of *HvGW2* antisense plants. *GIF1* (*GRAIN INCOMPLETE FILLING 1*) gene that encodes a cell-wall invertase required for carbon partitioning during early grain-filling (Wang et al. 2008). *GIF1* is responsible for grain weight reduction, ectopic expression of the cultivated *GIF1* gene with the 35S or rice Waxy promoter resulted in smaller grains, whereas over-expression of GIF1 driven by its native promoter increased grain production. These findings, suggest that *GIF1* is a potential domestication gene and that such a domestication-selected gene can be used for further crop improvement.

Establishing a reliable barley transformation technology is very important for the functional characterization of candidate genes and and the produced transgenic lines are subject for further studies.

This work is supported by the NAP_BIO_06-NEWSEEDS research grant.

Oberschall , Deák M, Török K, Sass L, Vass I, Kovács I, Fehér A, Dudits D, Horváth VG (2000) A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses, Plant J 24(4):437-446.

Song XJ, Huang W, Shi M, Zhu MZ, Lin H-X (2007) A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase, Nat Genet 39(5):623-630.

Tingay S, McElroy D, Kalla R, Feig S, Wang M, Thornton S, Brettel R (1997) Agrobacterium tumefaciens mediated barley transformation, Plant J 11:1369-1376.

Trifonova A, Madsen S, Olesen A (2001) Agrobacterium mediated transgene delivery and integration into barley under a range of in vitro culture conditions. Plant Sci 162:871-880.

Wang E, Wang J, Zhu X, Hao W, Wang L, Li Q, Zhang L, He W, Lu B, Lin H, Ma H, Zhang G, He1 Z (2008) Control of rice grain-filling and yield by a gene with a potential signature of domestication, Nat Genet 40(11):1370-1374.

Supervisor: Gábor V. Horváth
E-mail: nbetty@brc.hu

Molecular basis of the blood-brain barrier function

Péter Nagyőszi

Laboratory of Molecular Neurobiology, Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

One of the most important functions of the mammalian blood-brain barrier (BBB) is to restrict the free movement of different substances between blood and neural tissue, and it plays a key role in the homeostasis of the central nervous system. The principal components of the BBB are the cerebral endothelial cells that form a continuous monolayer and are interconnected with tight junctions and adherens

junctions. The tight junctions are composed of transmembrane proteins (occludins, claudins, junctional adhesion molecules) connected to junctional plaque proteins (*i.e.* ZO-1). The transmembrane proteins of the adherens junctions are the cadherins linked through catenins (alpha, beta, gamma) to the cytoskeleton.

The blood-brain barrier is involved in a large variety of pathological processes. Little is known about the effects of nicotine exposure on BBB function. We investigated the changes affecting the tight and adherens junction proteins by cigarette smoke components, especially nicotine and polyaromatic hydrocarbons (PAHs). 24h treatment of cerebral endothelial cells with relatively high concentration nicotine led to a decrease in occludin, cadherin and ZO-1 expression. Similar but less pronounced effects were observed after 24 h treatment with phenanthrene. Results of the immunofluorescent analysis confirmed western blot data. We also performed two dimensional electrophoresis in order to explore the cellular proteins responsive to nicotine and PAHs in brain endothelial cells. We observed different responses of the cells to both nicotine and phenanthrene treatment resulting in altered expression of shock induced proteins, metabolic enzymes, signaling molecules. This confirms the cerebral endothelium as being a target to cigarette smoke components (Hutamekalin et al. 2008).

From clinical point of view, because of the relative impermeability of the barrier many drugs are unable to reach the CNS in therapeutically relevant concentration, making the BBB one of the major impediments in the treatment of CNS disorders. A number of strategies have been developed to circumvent this problem. One of the successfully used methods to deliver drugs – especially antitumoral agents – to the CNS is the osmotic opening of the BBB using mannitol. This causes a rapid opening (within minutes) of the BBB which is reversible.

We investigated the effect of mannitol treatment on brain endothelial cells and found that mannitol induced a rapid, concentration dependent, and reversible tyrosine phosphorylation of a broad range of proteins between 50 and 190 kDa. One of the targets of tyrosine phosphorylation turned out to be the adherens junction protein beta-catenin and this phosphorylation was Src-kinase dependent (Farkas et al. 2005).

Beside beta-catenin and Src kinase, we aimed to find new signaling pathways activated by hypertonicity in cerebral endothelial cells and identified the receptor tyrosine kinase Axl to become tyrosine phosphorylated in response to hyperosmotic mannitol. Besides activation, Axl was also cleaved in response to osmotic stress. Specific knockdown of Axl increased the rate of apoptosis in hyperosmotic mannitol-treated cells; therefore, we assume that activation of Axl may be a protective mechanism against hypertonicity-induced apoptosis. Our results identify Axl as an important element of osmotic stress-induced signalling. (Wilhelm et al. 2008).

Farkas A, Szatmári E, Orbók A, Wilhelm I, Wejksza K, Nagyoszi P, Hutamekalin P, Bauer H, Bauer HC, Traweger A, Krizbai IA (2005) Hyperosmotic mannitol induces Src kinase-dependent phosphorylation of beta-catenin in cerebral endothelial cells. J Neurosci Res 80(6):855-861.

Hutamekalin P, Farkas AE, Orbók A, Wilhelm I, Nagyoszi P, Veszelka S, Deli MA, Buzás K, Hunyadi-Gulyás E, Medzihradszky KF, Meksuriyen D, Krizbai IA (2008) Effect of nicotine and polyaromtic hydrocarbons on cerebral endothelial cells. Cell Biol Int 32(2):198-209.

Wilhelm I, Nagyoszi P, Farkas AE, Couraud PO, Romero IA, Weksler B, Fazakas C, Dung NT, Bottka S, Bauer H, Bauer HC, Krizbai IA (2008) Hyperosmotic stress induces Axl activation and cleavage in cerebral endothelial cells. J Neurochem 107(1):116-126.

Supervisor: István Krizbai
Email: nagyoszi@brc.hu

Investigation of the maturation of NiFe hydrogenases in *Thiocapsa roseopersicina*

Andrea Nyilasi

Department of Biotechnology, University of Szeged, Szeged, Hungary

Our model organism, *Thiocapsa roseopersicina* BBS is an anaerobic, phototrophic purple sulfur bacterium. There are at least two membrane-bound (HynSL and HupSL) and one soluble (HoxEFUYH) [NiFe] hydrogenases in the cells. A typical [NiFe] hydrogenase is composed of a large and a small subunit. The large subunit harbors a specific NiFe catalytic metallocenter associated with CO and CN ligands (Volbeda et al. 1995.) The maturation of these complex enzymes require numerous accessory proteins. Most of these auxiliary genes were found using transposon mutagenesis, one of them was the *hupK* gene. (Maróti et al. 2003.) The product of this gene, the HupK protein is present only in organisms containing at least one membrane-bound [NiFe] hydrogenase enzyme.

The role of HupK is not known yet. In order to investigate the role of this protein, *ΔhupK* mutant strains were created, then the hydrogenase activities of the wild type and the mutant strains were compared. The results clearly showed that HupK protein is important for the formation of the functionally active membrane-bound hydrogenases, but not for the biosynthesis of the soluble enzyme. (Maróti et al. 2003.)

More detailed information can be obtained from biochemical experiments. Special expression vector was used to produce active, tagged HupK protein in homologous host. The tagged HupK protein was purified under mild conditions to retain all protein-protein interactions and the copurified proteins were analyzed by mass spectrometry. From cells grown under standard conditions, only one protein partner, namely the GroEL chaperonin could be fished out. The specific role of GroEL in the hydrogenase maturation is not likely, therefore alternative growth conditions were used to find the specific partners. Nickel starvation of the cells is supposed to result in the accumulation of the intermediates of the posttranslational process. Therefore, the tagged HupK protein was purified from such cells, however only one co-purifying band was observed: the PuhA protein, which is the H subunit of the photosynthetic reaction centre.

Metal content determination of the purified HupK protein from homologous host was performed. The HupK protein was shown to contain nickel atom in 1:2 molar ratio, and no Fe atom was detected in the sample. In order to determine, which amino acids assist in binding the nickel atom, the sequences of the HupK and the large subunits of the hydrogenases were compared. Conserved regions could be recognized at the N- and C-termini, while the middle part of the proteins was variable. The alignment uncovered two conserved cysteine residues as candidates for coordination of the metal. One of them is in the R-X-F-X-X-C motif at the amino terminus, other one is in the D-P-C-X-X-F motif at the carboxyl terminus. In order to examine the putative role of these residues, site-directed mutagenesis were performed and the effects of the mutations were monitored via the hydrogenase activties of the membrane-associated hydrogenases. A mutant carrying alanine instead of Cys378 had only 65% activity of the wild-type strain. However, the replacement of the Cys54 by alanine led to a considerable reduction in the hydrogenase activity (to 25% of the wild type level). Ni content investigation of the Cys54Ala mutant HupK protein revealed that it contains the same amount of Ni atom like the wild-type protein.

The R-X-C-X-X-C and the D-P-C-X-X-C sequences in the large subunits of NiFe hydrogenases have been shown to be essential for their activity and the cysteine residues have been proposed to form a coordination sphere surrounding the NiFe center (Przybyla et al. 1992, Volbeda et al.,1995.). In the HupK protein, phenylalanines subtitute the first and the last cysteins. In order to confer the motifs of the hydrogenas large subunit on HupK, the two phenylalanines were replaced by cysteines. The effect of these mutations on the biosynthesis of the membrane-bound hydrogenase is being investigated.

Maróti G, Fodor BD, Rákhely G, Kovács AT, Arvani S, Kovács KL (2003) Accessory proteins functioning selectively and pleiotropically in the biosynthesis of [NiFe] hydrogenases in Thiocapsa roseopersicina. Eur. J Biochem 270(10):2218-227.

Przybyla AE, Robbins J, Menon N, Peck HD Jr. (1992) Structure-function relationships among the nickel-containing hydrogenases. FEMS Microbiol Rev 8(2):109-135

Volbeda A, Charon MH, Piras C, Hatchikian EC, Frey M, Fontecilla-Camps JC (1995) Crystal structure of the nickel-iron hydrogenase from Desulfovibrio gigas. Nature 373(6515):580-587.

Supervisors: Gábor Rákhely, Kornél Kovács
E-mail: nyilasi@brc.hu

Anthropological Analysis of the Medieval Cemetery of 'Szeged-Vár'

Krisztina Prazsák-Hajnal

Department of Anthropology, University of Szeged, Szeged, Hungary

The environs of Szeged was a populated area from the primitive age; archeological finds prove there were Roman inhabitants at the time of Roman Empire, and later habitation during the great migrations of Huns, Gepids and Avars. This area was important because this is where Maros runs into the Tisza River, the Maros River being an excellent and cheap transport possibility of salt from Transylvania.

The first mention of the Medieval town is from 1246. As a result of the Turkish occupation of Hungary limited data is available, making the archeological excavation of Szeged-Vár very important.

The Medieval cemetery of Szeged-Vár was used from the Hungarian conquest to 1543, and from 1686 until 1713. The excavations have been going on since 1999, and by now approximately 700 graves have been excavated, along with some objects and crypts.

In this study, we have researched 425 graves excavated between 1999 and 2004. The basis of the anthropological analysis was the determination of sex and age of death, inclusion metric data, and paleopathological and taxonomical analysis. To determine these data, we have used common anthropological methods. Paleopathological and taxonomical examinations have been carried out using macromorphological methods, though in certain cases radiographical analysis was also applied.

After the determination of sex and age, we could establish that in this population the sex ratio was 50%-50%; the percentage of infants (INF1, INF2, JUV) was 49%, and elderly (SEN) 7%.

By means of the measurements of humerus, radius, ulna, tibia and femur we determined the height of people. The average of the height of adult males was 170 cm, adult females 160 cm; the highest was 181 cm both among males and females, the minimum height was 157.5 cm among males and 147.7 cm among females.

In accordance with general medieval health, many of the skeletons showed different forms of paleopathological lesions: periostitis, osteomyelitis, arthritis; minor developmental anomalies: sacralisation, lumbarisation, spina bifida, dislocation of the hip; traumas: fractures of humerus, radius, ulna, ribs or clavicle. There also were infectious bony lesions due to TB and syphilis. We found some metabolic disturbances of bone: osteoporosis; and circulatory and hematologic disorders as well: cribra orbitalia and cribra cranii.

The taxonomical analysis could yield some very interesting information because there is no such data available for this town. There are some finds in the area of a 'Kun' population (for example in Kiskundorozsma), and we suppose there was a Mongolid population in Szeged after the Hungarian conquest. For taxonomical analysis we have to research anatomical variations like sutura metopica, os Wormiana, torus palatinus, torus mandibulae and maxillae, fossa canina and several dental variations.

This presentation is for the preliminary results only.

Supervisors: Antónia Marcsik, György Pálfi
E-mail: aurorakr@citromail.hu

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Mitochondrial sequence variation in ancient horses from the Carpathian Basin and possible modern relatives

Katalin Priskin

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Whatever the place, ethnic changes always leave their footprint in the local culture and genetic makeup and the same applies to the different types of horses moving with their owners. This thesis is concerned with the mitochondrial control region genotypes of ancient and modern horses from the Carpathian Basin, where in the late 9th cenury the incoming pagan Hungarian tribes permanently changed the population.

Studies of mitochondrial DNA have shown that modern horses are descended from at least 77 different wild mares, with a last common ancestor over 300,000 years ago, and so probably inhabiting very different regions. Despite this matrilineal genetic diversity, correlations between modern breeds of horses and mitochondrial genotype are often uncommon. This is probably because horse-trading and horse-stealing, sometimes over long distances, have been popular and profitable for a long time.

To determine genetic diversity and origin of horse populations in the Carpathian Basin at the time of the Avars and of the Hungarian Conquest, mitochondrial DNA analysis was undertaken on 31 archaeological horse remains, excavated from authentic, well-dated Avar and pagan Hungarian burial sites. Based on a supposed relationship, modern Hucul and Akhal Teke horses were included in the analysis. To reveal relationships to other ancient and recent breeds, mtDNA sequences from 79 breeds representing 913 individual specimens were combined with our sequence data. Sequences were aligned and truncated to a length of 247 bp to accomodate published short sequences (nucleotide positions 15495-15740 of reference sequence X79547).

Estimation of standard diversity measures, such as haplotype diversity (h) and nucleotide diversity (π) were performed in DnaSP 4.50.2.

To compare our samples with other modern and ancient horse sequences, 921 previously published equine mtDNA CR sequences with fully overlapping standard 247 bp lengths were obtained from the database (http://www.ncbi.nlm.nih.gov/Genbank).

Median-joining network was constructed using the NETWORK 4.5.1 software (Fluxus Technology Ltd. at www.fluxus-engineering.com) to reveal approximate genealogical relationships among the haplotypes found in our ancient and recent breeds and the haplogroup-indicating sequences.

Both genetic distances and haploltype-based methods indicate a clear separation between horses of the Avar and Hungarian leading nobles. Avar sequences were genetically heterogeneous, closely related to Eastern breeds; (with Mongolian and north Russian Vyatskaya groups). This Asian relationship can also be seen in the genetic distance matrix and the haplotype network.

By contrast, beside the great heterogenity and unique haplotypes, the ancient Hungarian horses showed a relatively close relationship with the Turkoman Akhal Teke and Norwegian Ffjord. It confirms the assumption of Hecker (1955) and is an admixture to the clew up of the Hungarian origin. After a short communication of Bjørnstad (2003), different distance measures suggest genetic associations between northern European horse breeds and the Mongolian native horse. By all means, the high variability of Hungarian horse haplotypes may be connected with the well-attested, continent-wide raiding habits of the ancient Hungarians. The Hucul data show no such relationship. Our results show that the ethnic changes induced by the Hungarian Conquest in the late 9th century were accompanied by a similar change in the stables of the Carpathian Basin.

Supervisor: Raskó István
e-mail: priskin@brc.hu

Isolation and characterization of bacterium and phage isolates which have biocontrol ability against *Pseudomonas* strains pathogenic to *Pleurotus ostreatus*

Enikő Sajben

Department of Microbiology, University of Szeged, Szeged Hungary

Pleurotus ostreatus is one of the most extensively cultivated mushrooms in the world; however significant loss of crop and quality arises from bacterial diseases causing by different bacterial pathogens. The yellowing of *Pleurotus ostreatus* and the brown blotch disease of *Agaricus bisporus*, caused by *Pseudomonas tolaasii* is well known (Lee and Cha 1998). The bacterium produces the toxins tolaasins that disrupt the cellular membrane by forming pores (Rainey et al. 1992).

Ps. tolaasii can be identify easily, with tolaasin toxin gene specific primers. This method is much more reliable than the white line test. The *Ps. tolaasii* is able to make a phenotypic switch; this variant form is nonpathogenic and differs from the wild type in a range of biochemical and physiological characteristics (Cutri et al. 1984).

Moreover other fluorescent pseudomonads such as *Ps. agarici*, *Ps. constantinii* and *Ps. gingeri* can cause various symptoms as well. The same degree of discoloration may be caused by dissimilar species of pseudomonads, suggesting that the factors are not exclusive to a particular pseudomonad species (Godfrey et al. 2001).

Many investigations have been carried out to find an appropriate method for preventing or controlling this disease. There are trials to use of chemical wash formulations, including such chemicals as calcium chloride, sodium hypochlorite, hydrogen peroxide, bronopol and antibiotics in watering mushrooms, but none of them has been found to be fully effective and non-toxic to humans (Wong and Preece 1985).

Biological control by competition has also been investigated. Potential bacterial control agents have been isolated and shown to be active antagonists (Nair and Fahy 1972).

In this study, the *Pseudomonas* strains of an infected oyster mushroom farm in Hungary were investigated. Sixty strains were isolated on Gould's S1 medium, which consistently gives high selectivity and good recoveries of fluorescent pseudomonads with samples obtained from a variety of habitats. S1 medium has several advantages over other media used for the isolation of fluorescent pseudomonads. The identification of the strains was carried out by sequencing a part of the *rpoB* gene or in some other cases a part of the 16S rDNA gene. The *rpoB* gene codes for the RNA polymerase β-subunit, it is a highly conserved essential gene, so it could be used for bacterial identification. The pathogenicities of the strains were tested on yeast extract media in Petri-dishes, in direct confrontation tests.

To find and effective antagonistic agent, we isolated and evaluated fluorescent pseudomonads, bacilli and lytic bacteriophages, against *Pseudomonas tolaasii*, and other pathogenic *Pseudomonas* strains.

Cutri SS, Macaule BJ and Robert YWP (1984) Characteristics of pathogenic non-fluorescent (smooth) and non-pathogenic fluorescent (rough) forms of *Pseudomonas tolaasii* and *Pseudomonas gingeri*. J Appl Bacteriol 51:291-298.

Godfrey SAC, Harrow SA, Marshall JW and Klena, JD (2001) Characterization by 16S rRNA sequence analysis of pseudomonads causing blotch disease of cultivated *Agaricus bisporus*. Appl Environ Microb 67:4316-4323.

Lee H-I and Cha J-S (1998) Cloning of a DNA fragment specific to *Pseudomonas tolaasii* causing bacterial brown blotch disease of oyster mushroom (*Pleurotus ostreatus*). Kor J Plant Pathol 14:177-183.

Nair NG and Fahy, PC (1972) Bacteria antagonistic to *Pseudomonas tolaasii* and their control of brown blotch of the cultivated mushroom *Agaricus bisporus*. J Appl Bacteriol 35:439-442.

Rainey PB, Brodey CL and Johnstone K (1992) Biology of *Pseudomonas tolaasii*, cause of brown blotch disease of the cultivated mushroom. Adv Plant Pathol 8:95-117.

Wong WC and Preece, TF (1985) *Pseudomonas tolaasii* on mushroom *(Agaricus bisporus)* crops: bactericidal effects of six disinfectants and their toxicity to mushrooms. J Appl Bacteriol 58:269-273.

Supervisor: László Manczinger
E-mail: sajben@freemail.hu

Interacting protein partners on Drosophila telomeres

Sándor Takács

Department of Genetics, University of Szeged, Szeged, Hungary

The *proliferation disrupter* (*prod*) gene in *Drosophila* encodes a 346- amino-acid protein that localizes strongly to the centric heterochromatin of the second and third chromosomes as well as to >400 euchromatic sites, and all telomeres. In *Drosophila melanogaster*, three telomeric domains can be distinguished by DNA sequence and by proteins associated with them: the end of the chromosomal DNA molecule (capping complex), the retrotransposon array consisting of three non-long terminal repeat retrotransposons, *HeT-A*, *TART*, and *TAHRE* (HTT), and the subtelomeric repetitive telomere associated sequences, repetitive region (TAS). Chromosome length in *Drosophila* is maintained by targeted transposition of the three telomere-specific non-long terminal repeat retrotransposons, HTT, to the chromosome end.

Immunofluorescence stainings of different mutant telomeres clearly showed, that Prod binds to the *HeT-A* element of the HTT array. We could also show that Prod binding represses *HeT-A* transcription, nevertheless does not influence HTT length. Reduction of Prod levels in heterozygous $prod^{k08810}$ null mutant flies results in elevated levels of *HeT-A* transcripts in ovaries as well as in third instar larvae, while it has no effect on genomic *HeT-A* copy number, which we used as a measurement for HTT length.

To identify proteins that may interact with Prod, we performed a yeast two-hybrid screen, using the Clontech Matchmaker cDNA library and the entire *prod* cDNA as bait. About 120 000 cDNA clones were tested and about 100 potential Prod interactors were identified. Prod was found 4 times, suggesting that Prod interacts with itself. We also identified Z4 as one of the interacting proteins, which is one of the few proteins known to be associated with the HTT telomeric domain. The Chromator protein, which has previously been shown to co-immunoprecipitate with Z4 and co-localize with Z4 in interbands of polytene chromosomes and at some telomeres, was also found to interact with Prod. We could verify the latter interaction with co-immunoprecipitation experiment.

Proteins Uba2 and Lesswright were the strongest interactors of Prod in the yeast two hybrid screen.Uba2 is the E1 SUMO activating enzyme regulating the initial steps of sumoylation, - a posttranslational protein modification system modifying protein activity - while the second step is performed by the E2 ubiquitin-conjugating enzyme encoded by the lesswright gene in *Drosophila*. Computer sequence analysis showed that Prod (and Chromator) have potential sumoylation sites. This raised the possibility that either Prod itself is sumoylated or sumoylation enzymes are recruited by Prod to sumoylate Prod-associated proteins. Our immunostainings confirm this notion, demonstrating that most if not all sumoylated proteins on telomeres are located on the HTT array.

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

We tried to identify the sumoylation site(s) in Prod by disrupting each potential site one by one, and expressing the HA-tagged mutant proteins in transfected S2 cells. Next we immunoprecipitated the mutant proteins with anti-HA antibody, and tested their molecular weight and sumoylation on Western blots. In one of the mutant proteins the high molecular weight sumoylated band seemd to disappear, and the S2 cells expressing this protein showed an altered Prod chromosomal immunostaining pattern. This indicates that the Prod protein is sumoylated at the 123rd lysine aminoacid.

Török T, Benitez C, Takács S, Biessmann H (2007) The protein encoded by the gene *proliferation disrupter* (*prod*) is associated with the telomeric retrotransposon array in *Drosophila melanogaster*. Chromosoma 116(2):185-195.
Takács S, Szulik Á, Török T. The role of sumoylation on Drosophila telomeres. (manuscript in preparation).

Supervisor: Tibor Török
E-mail: takacssanyi@gmail.com

The role of the small GTPase LIP1 in the function of the plant circadian network

Kata Terecskei

Institute of Plant Biology, Biological Research Center, Szeged, Hungary

The circadian clock is a biological timing mechanism that provides rhythmicity to gene expression, metabolism, and physiology with ~24h periodicity. The central oscillator of eukaryotic clocks is based on the network of clock genes and proteins, which are interconnected by transcriptional/translational negative feed-back loops.

Current models of the plant circadian clock postulate three interlocked feedback loops. A pair of single Myb-domain transcription factors, *CIRCADIAN CLOCK ASSOCIATED1 (CCA1)* and *LATE ELONGATED HYPOCOTYL (LHY)*, plays central roles in two loops. In one loop, *CCA1* and *LHY* repress the expression of the Pseudo-Response Regulator gene *TIMING OF CAB EXPRESSION 1 (TOC1)*. *TOC1* closes the first loop by inducing *CCA1* and *LHY* transcription for the next cycle. In a second loop, *PRR7* and *PRR9*, are induced by *CCA1* and *LHY*. *CCA1* and *LHY* are subsequently repressed by *PRR7* and *PRR9*. In a third loop, *GIGANTEA (GI)* and, possibly, *PRR5* are positive regulators of *TOC1*. *GI* is negatively regulated by both *CCA1/LHY* and *TOC1* (McClung 2008).

The *lip1-1* (light insensitive period 1) mutant isolated from *Arabidopsis thaliana* displays novel circadian phenotypes. *lip1-1* was isolated as an early-phase mutant based on the expression pattern of *CAB2:LUC* circadian output marker in constant darkness. In wild-type plants, period length shortens with increasing light fluence rates and the phase of rhythms can be shifted by light pulses administered to darkadapted plants. In *lip1-1*, period length is nearly insensitive to light intensity and larger phase shifts can be induced during the subjective night (Kevei et al 2007).

The first aim of our work was to determine the molecular mechanism by which LIP1 affects the plant circadian clock. Transcript levels of clock genes were determined by quantitative real-time PCR in *lip1* mutants. Our data show that LIP1 affects the expression of *GI*, *PRR9* and *TOC1*. The effect on *GI* expression was supported by the analysis of *gi-lip1* double mutant plants.

We generated promoter:LUC+ reporter gene constructs for each core clock genes in *lip1* mutant background and we could prove that the transcription of all core clock components is affected by the mutation.

Our second aim was to identify how the function of LIP1 is controlled. LIP1 is a plant-specific atypical small GTPase. Small GTPases are molecular switches shuttling between the GDP-bound inactive and the GTP-bound active states. For this process they require downstream signaling elements (effectors) and upstream signaling elements (e.g. GEFs) (Berken et al 2005). We found that LIP1 interacts with a member of the plant specific family of RopGEFs, RopGEF7 in yeast two-hybrid system. However, the insertion mutant allele of *RopGEF7* showed no circadian phenotype in planta. The family of RopGEFs consists of 14 members. We tested the circadian phenotype of insertion mutants for all of them and found that a mutant allele of *RopGEF2* has a *lip1*-like circadian phenotype. *lip1* mutant plants show stress phenotype also, they are sensitive to salt. *RopGEF2* mutant plants display a *lip1*-like salt phenotype. RopGEF2 might be the member of the RopGEF family which promotes LIP1 function.

Previous data showed that LIP1 is localized in the cytosol, nucleus and in cell compartments as well. We tested the function of *nuclear export signal* (NES) or *nuclear localization signal* (NLS) tagged YFP-LIP1 fusion proteins in *lip1* mutant background to see if any of the *lip1* phenotypes could be complemented. YFP-LIP1-NLS fusion proteins could restore the circadian phenotype. Neither of the constructs could restore the salt sensitivity phenotype. These data indicate that LIP1 affects the circadian clock in the nucleus, but nucleo-cytosolic shuttling is required to fulfill its role in tolerating salt stress.

Berken A, Thomas C, Wittinghofer A (2005) A new family of RhoGEFs activates the Rop molecular switch in plants Nature 436:1176-1179.
Kevei É, Gyula P, Fehér B, Tóth R, Viczián A, Kircher S, Rea D, Dorjgotov D, Schafer E, Millar AJ, Kozma-Bognár L, Nagy F (2007) Arabidopsis thaliana Circadian Clock Is Regulated by the Small GTPase LIP1 Current Biology 17:1456-1464.
McClung CR (2008) Comes a time. Current Opinion in Plant Biology 11:514-520.

Supervisors: László Kozma-Bognár and Ferenc Nagy
E-mail: terecs@brc.hu

Volume 53(1):55-76, 2009
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Progress report. Investigating the neuroprotective function of Hsp27

Melinda Erzsébet Tóth

Institute of Biochmeistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Hsp27 is expressed in many cell types and tissues at specific stages of developement and differentiation. It is an ATP independent chaperone, its main function is to bind misfolded proteins hereby block the formation of protein aggregates. The protein can protect cell against stressful stimuli through its several other properties. For example it has been shown to modulate apoptosis since it can interact with and inhibit key components of the apoptotic signaling pathway. Hsp27 can also increase the resistance of cells to oxidative injuries as it reduce lipid peroxidation, protein oxidation and f-actin architecture disruption.

In order to study the neuroprotecive effects of Hsp27 in vivo, transgenic mouse lines overexpressing the human Hsp27 were generated. The human Hsp27 protein expressed at high level in the brain of the transgenic mice demonstrated by western blot analysis. The cellular localization of the expression of transgenic protein in the brain was detected by immunohistochemistry. We found strong expression of the transgene in the cerebellum, hippocampus and cerebral cortex.

The neuroprotective effect of Hsp27 protein was investigated in acute and chronic ethanol administration. In the acute administration studies animals received an intraperitoneal injection of 20% ethanol (2g/kg) than behavioural studies were performed to analyse motor coordination and muscle strength. Five different behavioural tests were performed and in three of the tests statistical analysis using one-way analysis of variance (ANOVA) revealed a significant difference in the performance of transgenic and wild type mice. In the inverted screen test all of the wild type animals showed ataxia compared to the transgenic group, where it was significantly less (57%) (p=2,925E-4). In the beam walking test 86% of wild type mice fell off the beam, while in the transgenic group only 53% (p=0,031). In the footprint analysis we found significant difference in the stride length between the transgenic and wild type groups (p=0,002 for the forlimb and p= 5,6036E-6 for the hindlimb).

In the chronic ethanol treatment drinking water was replaced by a 20% of ethanol solution for five weeks. At the end of the treating period 10 μm frozen sections were made from the brain of the animals. Neurodegeneration was detected by Fluoro-Jade C staining. We detected less degenerated neurons in the brain of the transgenic mice compared to wild type mice especially in the cerebellum, hippocampal region and cerebral cortex.

Supervisor: Miklós Sántha
E-mail: tothe@brc.hu

The role of dADA2b adaptor proteins in dSAGA histone acetyltransferase complex

Nóra Zsindely

Department of Biochemistry and Molecular Biology, University of Szeged, Szeged, Hungary

In eukaryotes the genetic material is present in a compact chromatin structure consisting of DNA and histone proteins. Histone acetyltransferase (HAT) complexes play a role in chromatin structure modifications which might lead to changes in gene expression. The GCN5 protein is the catalytic component of several multiprotein HAT complexes which modify chromatin structure by acetylating specific lysine residues at the N-terminal tails of histone H3 and H4. Many of the GCN5-containing HAT complexes also contain ADA-type adaptor proteins, which play roles in modulating HAT activity and specificity.

In *Drosophila melanogaster* our group has described two ADA2 proteins (dADA2a and dADA2b) in two GCN5-containing HAT complexes, ATAC and dSAGA which have different histone specificities. The dADA2b-containing dSAGA complex is involved in the post-translational modification of nucleosomal histone H3 at lysine (K)9 and K14. Furthermore, analysis of the *dAda2b* gene revealed that by alternative splicing it gives rise to two mRNAs (dAda2bS and dAda2bL). Despite the detection of two different forms of dAda2b message in earlier studies, the production and function of the two protein isoforms have not been studied.

In my thesis work I have shown that *dAda2b* produces at least two protein isoforms, dADA2bL and dADA2bS during the course of development and demonstrated that dADA2b proteins are most abundant in pupae, in the developmental stage when in the absence of the proteins *dAda2b* null mutants die.

My further studies included generation of *dAda2b* mutations which eliminate the production of either or both dADA2b isoforms in order to characterize dADA2b function in dSAGA complexes. Functional studies of *dAda2b* null mutant reported so far demonstrated the essential function of *dAda2b* gene in Drosophila development and the requirement for histone H3K9 and K14 acetylation. Genetic studies showed that neither dADA2b isoform alone could provide a complete restoration of *dAda2b* function, suggesting that both are required for development. On the other hand, either dADA2b isoform can productively participate in dSAGA complexes and render those at least partially active in histone H3 acetylation.

To study the molecular consequences of the loss of H3 K9 and K14 acetylation we compared the mRNA profiles of wild-type and *dAda2b* mutant animals in late L3 larval and in P4 pupal stages by cDNA microarray. Global gene expression profiling indicates that the

expression of less than 5% of the genes is significantly changed in *dAda2b* null mutants at both time points analyzed. We observed a relatively small overlap between the two stages in both mutant and wild-type samples. More than half of the affected genes are up-regulated, of which the high number of defense-related genes is particularly striking.

To demonstrate whether dSAGA has a direct role in the regulation of those genes which showed altered transcription levels in *dAda2b* mutant, we performed chromatin immunoprecipitations (ChIP) to detect the presence of dSAGA-specific histone H3 acetylation marks on these genes. Interestingly, we found that *dAda2b* mutation affects the H3K9ac level by a different extent at dSAGA-dependent and independent genes. In *dAda2b* mutants the histone acetylation levels are decreased both at dSAGA up-regulated, and at dSAGA down-regulated genes, while in the promoters of dSAGA independent genes a high level of histone H3K9ac is maintained.

Our data support the notion that different dADA2 proteins contribute to the functional versatility of multiprotein complexes and demonstrate that the loss of dSAGA specific H3 K9 and K14 acetylation affects (up- or down-regulates) the expression of a rather small subset of genes but does not cause a general transcription deregulation. We envision that the loss of *dAda2b* function results in a global decrease in H3 acetylation which can lead to an increase or to a decrease in the transcription level of genes.

Zsindely N, Pankotai T, Ujfaludi Zs, Lakatos D, Komonyi O, Bodai, L, Tora L, Boros I. The loss of histone H3 lysine 9 and lysine 14 acetylation due to dSAGA-specific *dAda2b* mutation influences the expression of only a small subset of genes. Submitted to Nucleic Acids Research, in revision.

Supervisor: Imre Boros
E-mail: zsindely@bio.u-szeged.hu

Instructions to Authors

Submission of manuscripts

Submission of a manuscript to Acta Biologica Szegediensis automatically involves the assurance that it has not been published and will not be published elsewhere in the same form. Manuscripts should be written in English. Since poorly-written material will not be considered for publication, authors are encouraged to have their manuscripts corrected for language and usage by a trusted expert.

There are no explicit length limitations: a normal research article will occupy 4-6 printed pages; reviews might be considerably longer. Authors should submit three sets of the complete manuscript and illustrations, together with a computer disk containing an electronic version of their manuscript. The elctronic file is considered the final material. Both Macintosh and PC versions will be accepted. The disk should be labeled with the date, the first author's name, the file name of the manuscript and the software, disk format and hardware used. Acta Biologica Szegediensis will not return copies of submitted manuscripts and figures. Requests to return original figures will be honored as a courtesy, but cannot be guaranteed. If instructions are not followed, authors will be asked to retype their manuscripts.

Manuscript format

Only good-quality laser printouts will be accepted. All pages should be printed with full double spacing, 2.5 cm margins, and a nonjustified right margin. A standard 12 point typeface (e.g. Times, Helvetica or Courier) should be used throughout the manuscript, with symbol font for Greek letters. Boldface, italics or underlined text should not be used anywhere in the manuscript. Footnotes are not permitted. Each page should be numbered at the bottom as follows:

Page 1. Title page: Complete title, first name, middle initial, last name of each author; where the work was done (authors' initials in parentheses if necessary); mailing address, phone, fax, and e-mail of the corresponding author; a running title of no more than 48 characters and spaces.

Page 2. Abstract: no more than 200 words, followed by 4-6 key words.

Beginning on page 3: Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, Figure Legends, Tables. Each section should be begun on a new page.

The manufacturer's name and location should be given in parentheses for reagents and instruments. Sources for all antibodies and nucleotide sequences should be indicated. Customary abbreviations in common use need not be defined in the text (e.g. DNA or ATP). Other abbreviations should be defined the first time that they are used. Quantitative results must be presented as graphs or tables and supported by appropriate experimental design and statistical tests. Only SI units may be used. For studies that involve animals or human subjects, the institutional, national or international guidelines that were followed should be indicated.

References

Only work that has been published or is in the press may be referred to. Personal communications should be acknowledged in the text and accompanied by written permission. In the text, references should be cited by name and year, e.g. Bloom (1983) or (Schwarz-Sommer et al. 1990) or (Maxam and Gilbert 1977). In the References, references should be listed alphabetically by first authors (including all co-authors) and chronologically for a given author (beginning with the most recent date of publication). Where the same author has more than one publication in a year, lower case letters should be used (e.g. 1999a, 1999b, etc.). Periods should not be used after authors' initials or abbreviated journal titles (e.g. Acta Biologica Szegediensis should be cited as Acta Biol Szeged). Inclusive page numbers should be used. Examples:

Bloom FE (1983) The endorphins: a growing family of pharmacologically pertinent peptides. Annu Rev Pharmacol Toxicol 23:151-170.

Coons AH (1978) Fluorescent antibody methods. In Danielli JF, ed., General Cytochemical Methods. Academic Press, New York, 399-422.

Maxam AM, Gilbert WA (1977) A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560-564.

Monod J, Changeux J-P, Jacob F (1963) Allosteric proteins and cellular control systems. J Mol Biol 6:306-329.

Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H (1990) Genetic control of flower development by homeotic genes in Antirrhinum majus. Science 250:931-936.

Illustrations

Three complete sets, including a high-quality "original" for publication, must be submitted with the manuscript. The back of each figure or composite plate should be labeled in soft lead pencil, indicating the orientation, the figure number, and the first author's name. The back of the best set should be marked "use for reproduction" or "original". Authors are encouraged to submit digital images of photographs, line drawings or graphs for printing. Most major image editing and drawing/illustrator computer software files (both Macintosh and PC) in TIFF or EPS formats are acceptable. It is particularly important that adequate resolution (at least 300 dpi, preferably 600 dpi) is used in making the original image.

Figure legends

Figures should be numbered consecutively with Arabic numerals. Material in the text should not be duplicated and methods should not be described. The size of scale bars should be indicated when appropriate. The first figure in the text should be referred to as Fig. 1, and so on.

Tables

Tables should be numbered consecutively with Arabic numerals. A brief title should be included above the table. Each table should be printed double spaced, without vertical or horizontal lines, and on a separate sheet. Material in text should not be duplicated and methods should not be described. The first table in the text should be referred to as Table 1, and so on.